In vitro methods for the construction of chimeras in potato

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IN VITRO METHODS FOR THE CONSTRUCTION OF CHIMERAS IN POTATO

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ABBREVIATIONS

Growth Regulators

2,4-D = 2,4-Dichlorophenoxyacetic acid

BAP; B = 6-Benzylaminopurine

KIN; K = Kinetin (6-furfurylaminopurine)

IAA = Indole-3-acetic acid

NAA; N = α-Napthalene acetic acid

GA_3 = Gibberellic acid

Basal Medium

MS = Murashige and Skoog medium (1962)

Callus induction media

ST = Shepard and Totten (1977)

SCH/C = MS + growth regulators + sucrose

WO = " " "

AC = " " "

JCB = " " "

Regeneration media

M1 = MS + growth regulators + sucrose

M2 = " " "

E31/E41 = " " "

E32/E42 = " " "

D1/D2 = Shepard (1980) but with modifications recommended by Nelson et al., (1983)

SCH = Schumann et al., (1980)

Grafting techniques

2n→4n = diploid donor tissue grafted onto tetraploid recipient tissue

4n→2n = tetraploid donor tissue grafted onto diploid recipient tissue

AG = autograft

HG = heterograft

P.T.F.E. = polytetrafluorethylene tape

Other terms

2n = diploid

4n = tetraploid

cv = cultivar

pers. comm. = personal communication

AC = activated charcoal

μ = microns

$\bar{\chi}$ = mean

L1 = outer tunica

L11 = inner tunica

L111 = corpus

SUMMARY

The in vitro construction of periclinal chimeras from tissues of different species of potato has been studied. Two potential chimeral systems were investigated: a thin cell layer grafting system and a mixed callus system.

Thin cell layer grafting involved the excision of an epidermal layer (three to six cells) from an internodal stem section and then the replacement of this layer onto a stem section from which a similar epidermal layer had been removed, and which was derived from another species of potato (heterograft). Successful heterografts were achieved on MS basal medium containing equal concentrations of NAA and BAP ($2.0mgl^{-1}$). An 83% success rate was the maximum achieved with heterografts, but this varied according to the species combinations.

7% shoot regeneration was achieved with heterografts, root regeneration being the favoured response. However, from this relatively low percentage of organogenically competent heterografts, 161 shoot-tips were isolated. Of the 16 heterografts producing shoots 81% were composed of diploid donor tissue grafted onto tetraploid recipient tissue. 87% of the heterografts exhibiting organogenic response were cultured on MS basal medium containing zeatin riboside ($4mgl^{-1}$).

MS basal medium supplemented with 2,4-D ($2.0mgl^{-1}$) was found capable of inducing and maintaining callus from both the diploid and tetraploid species. Callus derived from stem tissue of diploid and tetraploid origin was combined using a number of treatments to produce mixed callus systems which were transferred

at intervals to regeneration media. Maximum organogenic response was achieved with both single callus and mixed callus systems using a two-stage procedure. This procedure utilised two media: medium one containined NAA ($0.1mgl^{-1}$), BAP ($0.5mgl^{-1}$) and $0.25gl^{-1}$ sucrose (w/v) whereas medium two contained IAA ($0.1mgl^{-1}$), zeatin ($1.0mgl^{-1}$) and $2.5gl^{-1}$ sucrose (w/v). Percentage shoot regeneration varied with species and cultivar examined but the optimum response was expressed by callus derived from a tetraploid species (70%). Maximum shoot regeneration from callus derived from a diploid species was 20%. Maximum shoot regeneration obtained from a mixed callus system was 50%.

255 shoots derived from both the thin cell layer grafting system and the mixed callus system were subjected to analysis aimed at determining the ploidy levels of the L1 and L3 layers. Observations were made concerning morphological differences *in vitro* and *in vivo* (potential chimeral shoots were established in soil and growth was continued *in vivo*), but decisions concerning potential chimeral structures were made largely on the basis of cytological analysis. Such analysis consisting of stomatal length measurements and chromosome numbers in root-tips, both *in vitro* and *in vivo*, led to the selection of six plants as potential periclinal chimeras. Chromosomal analysis revealed the presence of a relatively small number of aneuploids amongst the regenerants (5%).

CHAPTER I

INTRODUCTION

1.1. THE VALUE OF POTATO AS A FOOD CROP

The potato, one of mankind's most valuable foods, is produced in 130 countries where seventy-five per cent of the world's population live. As a food crop, potato has far greater potential in developing countries than is generally realised. On a per-hectare basis, it can produce more energy and utilizable protein than any other major food crop (CIP Information). Due to the high quality of its protein the potato can play an important role in balancing nutritionally poor diets, and furthermore, as land scarcities become more acute in developing countries, it is logical that the potato will be increasingly relied upon as a source of energy.

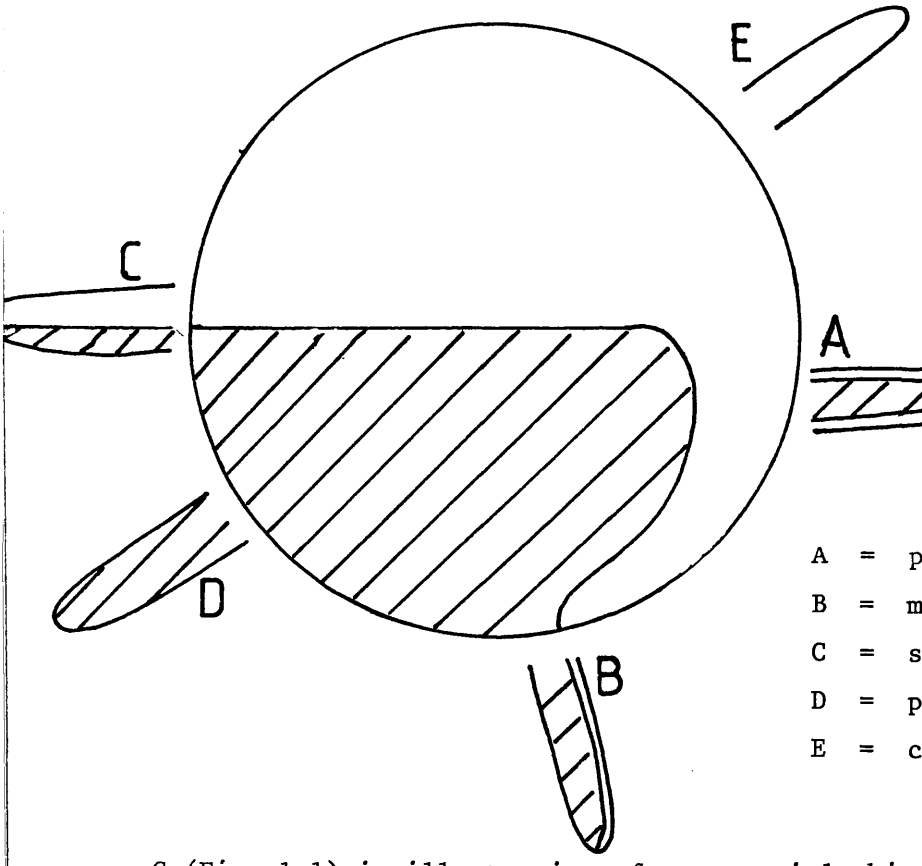
The potato is becoming an increasingly attractive food crop in developing countries, and in recent years potato production has spread gradually out of its traditional mountainous environment into warmer, generally drier, areas. However, the potato is susceptible to many viral pathogens, and virus diseases have been documented to cause up to eighty percent loss in yield. Control of virus diseases can be more problematic in developing countries than developed countries. Because potatoes are propagated vegetatively, seed tuber production normally cannot be isolated from consumer production areas and the control of virus transmission is lacking or erratic. The most important potato viruses are potato leafroll virus (PLRV) and potato viruses X and Y (PVX and PVY). PLRV and PVY, which generally occur together, are the most serious, being transmitted easily by aphids, even with low aphid populations. Consequently, there is a constant search for additional sources of general resistance to viruses.

Glandular trichomes of wild Solanum species are of interest in this respect, because of their role in resistance to insects and mites (Gibson et al., 1977; Tingey et al., 1978). For example, Solanum berthaultii is a wild tuber-bearing potato, notable for its resistance to a wide range of pests. Such resistance has been connected with the abundance of glandular trichomes covering its leaves and stems. Two types of glandular trichomes are associated with this resistance, a short type with a four lobed head (type A) and a longer multicellular type with an ovoid gland at its tip (type B). The two types of trichomes interact to confer higher levels of resistance to aphids, and accessions bearing both types demonstrate higher levels of resistance to the green peach aphid, Myzus persicae, Sulzer, and the potato leafhopper, Empoasca fabae Harris, than accessions bearing type A hairs alone (Mehlenbacher et al., 1983). Recent work by Gibson et al., (1983) has demonstrated that the exudate of type B trichomes is the alarm pheromone of most species and is released in sufficient amounts to disperse aphid colonies. This release is not a result of contact between aphids and plant tissue, for as Gibson et al., (1983) reports, aphids are repelled at a distance of 1-3mm from leaves of the wild potato Solanum berthaultii Hawkes.

1.2. PLANT CHIMERAS

The question has been raised as to whether these properties and other epidermis-specific defence mechanisms could be readily transferred from one variety to another by the construction of periclinal chimeras. The term chimera was given to plants composed of two genetically distinct tissues by Winkler (1907) after his grafting experiments had resulted in the production of a shoot divided longitudinally into two halves, one half was composed of tomato and the other of nightshade. Different types of chimeras exist and it is the position of the buds, with respect to internal tissues, which determines the nature of the chimera. Fig. 1.1. shows a section cut through a stem composed of two different tissues and illustrates the different chimeral forms which can arise depending on the position of the bud on the stem. A illustrates a periclinal chimera where the bud originates from that part of the stem where a strip of one tissue (in this case, colourless) overlaps the other (in this case, green). Thus, with a periclinal chimera the mutant lineage is usually confined to the tunica layer, in which the mutated cell arose. A mericlinal chimera is often considered as an incomplete periclinal chimeral, arising from a stem where the overlap of one tissue on the other is of small extent. The mericlinal shoot develops towards the edge of the wide overlap, and as shown by B (Fig. 1.1.), results in a shoot with colourless tissue running down the side of an otherwise green branch.

Fig. 1.1. Section cut transversely through a stem composed of two different tissues



A = periclinal chimera
B = mericlinal chimera
C = sectorial chimera
D = pure green shoot
E = colourless shoot

C (Fig. 1.1) is illustrative of a sectorial chimera, which is formed when the mutation occurs in a species with a monoplex or simplex apex, or early in embryo development, that is, when the apex is lacking in distinct layers. The mutant lineage forms a solid sector (colourless as in C) in the side of the shoot. Periclinal and mericlinal chimeras can only occur in species with a duplex apex, that is, an apex with two superimposed zones of growth, the tunica and corpus, each taking its origin from initials for each zone. Only anticlinal divisions occur in the one or more tunica layers, whereas divisions

in various planes contribute to the growth of the corpus.

A periclinal chimera was produced with an epidermis of Solanum pennellii Correll and a core of Lycopersicon esculentum Mill. The former was susceptible to greenhouse whitefly and potato aphid whereas Solanum pennellii was resistant to both. The resultant periclinal chimera was highly resistant to greenhouse whitefly and susceptible to potato aphid (Clayberg, 1974). Jørgensen (1928) attempted to put an outer layer of tomato resistant to potato late blight (Phytophthora infestans) onto a core of susceptible potato (Solanum tuberosum ssp. tuberosum), in the hope of obtaining resistant chimeral potatoes. However, Jørgensen's method for chimera production depended on the ability of both potato and tomato to develop callus and regenerate shoots from that callus, and as potato showed a reluctance to produce either to the required degree, Jørgensen was unable to produce the desired type of chimera.

The only persistent chimeras are periclinal ones. This stability is due to the independence of the layers in the apical meristem of the shoot. The one or more peripheral layers (tunica) are maintained almost exclusively by anticlinal divisions, whereas in the corpus, divisions are in random planes. Each layer in the apical meristem is destined to give rise to specific tissues within the plant; cells of the peripheral layer (L1 layer) produce the epidermis, those of the inner layers (L2 and L3 layers), the internal cortex and vascular system. Kupfer (1907) stated that in a potato three independent layers are present, whereas Howard (1963) concluded that L3 does not have an independent existence and he suggested that the apex

of axillary buds is produced only by L1 and L2. Furthermore, interchange of cells between these two layers is extremely rare or entirely absent (Howard, 1959). Howard (1961a and 1961b) also suggests that the number of initial cells is probably very small, six cells for L1 layer and two cells for L2 layer. Leaf shape in potato is mainly determined by L2 layer, any effects of the L1 and L3 layers on leaf shape are smaller than that of the L2 layer. The further upward growth of the leaf depends primarily on the activity of the cells originating from L2, L1 only contributing the epidermal layer of cells and the contribution of L3 being confined to the lower regions. This conclusion was primarily expressed by Satina (1959) as a result of work carried out with Datura and was later agreed with by Howard et al., (1963) after an investigation into leaflet shape of chimeras of Solanum x juzepczukii.

1.2.1. Formation of chimeras

The origin of most chimeral plants has been through spontaneous somatic mutation. Such chimeral forms have been maintained over many years because of the indeterminate growth of the vegetative apical meristem. Within the group of florist crops, several commercially important colour sports, shown to be chimeras, have originated as somatic mutants, for example "William Sim" carnation (Melquist et al., 1954), "Indianapolis" chrysanthemum (Stewart et al., 1970) and many Poinsettia cultivars (Stewart et al., 1966).

Another source of chimeras arises through the doubling of chromosome number in certain layers with colchicine or other chemical or shock treatments. Cells at different stages of the division

cycle are differentially sensitive, so that in a treated apical meristem only a limited number of cells are affected. If certain cells are affected and continue to divide, a cytochimeral form may be established and persist (Burk, 1975). Such induced or spontaneous mutations result from random genetic events and as such, rarely produce desirable changes, hence the usefulness of many spontaneous or induced chimeras is limited.

Spontaneous production of chimeras has occurred from graft unions. For example, the grafting of a scion of sour orange onto a seedling stock of citron in 1644 in Florence resulted in the development of a plant which bore fruit composed of a core of citron and an epidermal skin of orange (Neilson-Jones, 1969). Similarly, in 1825 in Paris, a graft between laburnum and broom gave rise to a number of buds and shoots, one of which grew more upright and vigorous, and with larger leaves than the usual broom. Examination of the structure of the plant revealed a core of laburnum covered by a skin derived from broom and hence the plant was known as Laburnocytisus adamii (Neilson-Jones, 1969). In 1907, Winkler grafted tomato and nightshade together and succeeded in obtaining adventitious shoots which were intermediate in form between the stock and scion. Winkler achieved this through removal of the scion from just above the graft union and as a result of this excision, callus developed from which adventitious shoots regenerated. The original chimera obtained by Winkler was a sectorial chimera (Fig. 1.1, Section 1.2), however shoots which developed later were of a periclinal nature.

Other workers have produced and described a number of graft chimeras of several Solanaceous species (Neilson-Jones, 1969). The periclinal chimera between Solanum pennellii Correll and Lycopersicon esculentum Mill was produced using a grafting technique (Clayberg, 1974). Clayberg used a simple approach graft with Solanum pennellii as the scion and Lycopersicon esculentum as the stock. He followed Krenke's technique (1933) to obtain chimeras, in which, by a cut parallel to the graft union, all the scion is removed except for a thin layer. From this cut surface callus developed, followed by regeneration of adventitious shoots.

Several attempts have been made to synthesise chimeras from mixed cell cultures (Ball, 1969; Koenigsberg et al., 1978; Carlson et al., 1974; Marcotrigiano et al., 1984). Carlson et al., (1974) isolated twenty-eight chimeral plants from approximately seven thousand callus derived shoots. The 'mixed' callus cultures (cultures consisting of callus derived from different species) were composed of Nicotiana tabacum and the amphiploid hybrid of Nicotiana glauca x Nicotiana langsdorfii. A medium was selected so as to favour regeneration of only one species and those shoots displaying the morphological characteristics of the species unable to regenerate were selected and tested to determine if they were chimeras.

Recent work by Marcotrigiano and Gouin (1984) used green and albino cell lines of Nicotiana species in mixed cell cultures of differing types. From these mixed cell cultures, 1321 plants regenerated and of these, four were chimeral, of which, three had originated from mixed cell suspensions, one from mixed callus fragments and none

survived *in vivo*. Several reasons for the production of such a small number of chimeras are discussed by the authors. Firstly, there is the suggestion that the production of so few chimeras could be a response partly determined by the low number of cells involved in shoot formation, the argument being that the larger the number of cells involved in this process, the more likely it is that cells of different lineage will be able to intermingle, and thus be able to participate, with respect to position, in shoot formation. If the assumption is made that shoots originate from very few cells, then the highest percentage recovery of chimeras should result from a cell culture where the greatest intermingling of cell types has been encouraged; in addition, lengths of time in culture should also influence the degree to which the genotypes intermingle. Carlson *et al.*, (1974) studied mixed cell cultures over a period of six months and concluded that "with each successive transfer, the two cell types which compose the callus became more interdispersed". Ball (1969), however, concluded from his study of mixed callus cultures that "there was no separation of individual derivative cells and infiltration of the general tissue mass. Thus ... derivative cells remained together making contributions to the tissue mass of an individual component of the mixture". Marcotrigiano *et al.*, (1984) cultured mixed cell lines for periods of eight to twelve weeks, possibly too short a length of time with which to achieve sufficient interdispersion of genotypes for the regeneration of chimeral shoots.

If, on the other hand, adventitious shoots are ultimately derived from single cells, non-chimeral shoots would be expected from

mixed cell cultures. However, whether or not adventitious shoots are derived from one cell or several cells remains controversial. Experimental evidence exists supporting both views and it is possible that the process is variable and differs according to the species of plant involved. Norris et al., (1983) studied adventitious shoot formation from leaf tissue of African violet chimeras and found that large numbers of chimeral plants were regenerated rather than the all-green or all-white plants that might have been expected. These authors also obtained three different periclinal chimeras with different shoot apex organization, which they suggested could only result if all three histogens participate in the development of adventitious shoots from leaf tissue. Adventitious shoot development in Nautilocalyx lyncheii has been demonstrated to be the result of simultaneous division of several adjacent epidermal cells (Tran Thanh Van et al., 1971). Bud formation in Torenia fournieri epidermis was seen to be the result of active cell division in single cells followed by division in two or three adjacent cells (Chylah, 1974). A histological study of adventitious shoot formation from leaf petiole cuttings of Rieger Begonia cv Aphrodite Peach produced results which suggested that adventitious shoots arise from a number of cells which may or may not originate from one epidermal cell of the petiole (Mikkelsen et al.,1978).

The major argument concerning the origin of adventitious shoots results from mutation studies where the irradiation of adventitious buds leads to the formation of solid non-chimeric mutants and unmutated plants with a very low proportion of chimeric plants. Broertjes et al., (1980) constructed a hypothetical model which they used to describe apex formation in adventitious buds. This model made it possible to estimate the percentage of chimeral shoots expected

as a result of mutation experiments with various crops, either propagated *in vivo* or *in vitro*. However, they found that the actual numbers of chimeras resulting from such experiments were lower than might have been expected from their hypothetical model. Thus, they postulated that either the apices of adventitious shoots were formed from a single epidermal cell of the meristem or callus, or that more than one cell was involved, but that apex formation was less of a random process than might have been thought. With respect to the latter, this could be a process of diplontic or intra-individual selection, whereby a particular cell, possibly the first dividing cell, assumes the dominant position within the meristem, becoming surrounded by genetically identical, daughter cells.

This diplontic selection could occur during meristem or callus formation by original epidermal cells and/or during apex formation by initial cells on top of the meristem or callus. Such a process could explain the high percentage of non-chimeric plants resulting from mutation experiments as it would lead to the elimination of all but one cell type in each of the histogenic layers. Broertjes (1972) argues against the possibility of diplontic selection occurring, basing his argument on evidence obtained from mutation experiments, with *Saintpaulia ionantha* in which it was found that dwarf mutants occurred even at low levels of radiation. He proposes that at such low levels, most cells were probably not mutated and would presumably be more vigorous than the dwarf mutant cells and therefore capable of suppressing the mutated cells, if diplontic selection were taking place. Further support for this argument was obtained through treating

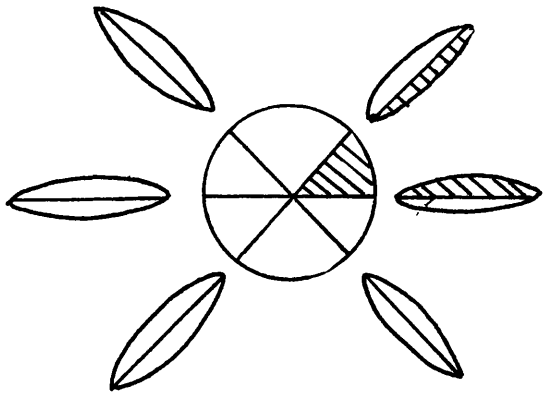
detached leaves of Saintpaulia ionantha with colchicine (Broertjes, 1972b). A high proportion of solid, non-cytochimeric tetraploids were present among the adventitious plantlets regenerated from the leaves, and consequently Broertjes argued that if diplontic selection was taking place it would be in favour of the diploids with the faster division rate. On the other hand, Mikkelsen et al., (1978) offer an alternative viewpoint. They carried out experiments involving irradiation of leaf petiole cuttings of Begonia x hiemalis Fotsch, the outcome of which was a lower percentage of chimeric plants, than might have been expected, among the plants which regenerated from the cuttings. Consequently, the authors (Mikkelsen et al., 1978), see diplontic selection as being operational during adventitious shoot formation, removing all but one cell type in each of the histogenic layers, resulting in the production of a genetically homogenous plantlet which may or may not have originated from a genetically homogenous group of cells.

A form of diplontic selection is also offered as the explanation for results obtained by Moh (1961), when irradiation of coffee seeds produced mutant plants irrespective of the radiation dose. Tilney-Bassett (1986) suggests that the irradiation treatment possibly inhibited co-ordinated division of the apical initials and consequently they might then be replaced by the first cell among them or within close proximity, that divided, and if that cell had undergone a mutation, the whole growing point would have become mutant. Thus the growing point would have behaved as if originating from a single initial, but this need not necessarily be the case in a situation

where irradiation is not used, and where more than one initial might be the norm.

Work by Howard (1966b; 1971b) aimed to clarify the situation with potato. A sectorial chimera of potato cv. Majestic in which one half of the leaf was entire and the other half, pinnate, suggested that such leaves originated from two initials in the inner tunica (Howard, 1966b). In contrast, another sectorial chimera composed of green and yellow tissue, bore leaves wholly of one colour, as well as individual half-and-half leaves; thus implying a minimum of six initials (see Fig. 1.2).

Fig. 1.2. Model showing the relationship between the genotype of cells within a group of apical initials and the development of the leaves



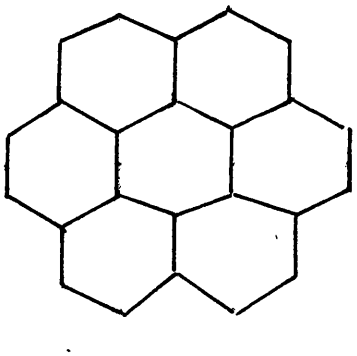
Six initials and a five sixths green, one sixth white sectorial chimera

However, to infer that the tunicas of potato shoots may have a variable number of initials, that is, two, three or six, contradicts the idea that the initial group is a fixed and stable structure. Thus,

Tilney-Bassett (1986) proposes that what is more likely is the existence of a constant number of initials which can be split in different ways, so six initials can accommodate a half and half sectorial (3:3 split), a one third, two thirds sectorial (2:4 split) and a one sixth, five sixths sectorial (1:5 split) without any change in the size of the initial group. Support for six initials comes from work by Stewart et al., (1970a) on a number of variegated plants which showed change in the size of sectors from $\frac{1}{2}:\frac{1}{2}$ to $\frac{1}{3}:\frac{2}{3}$.

Tilney-Bassett (1986) suggests that further support for six initials comes from the shapes of cells. Accepting that cells within a tissue appear to the eye as a pattern of linked polygons constructed according to basic laws (Dormer, 1980), an impression of the initial cells within a tunica is derived by viewing the tissue from above, or in T.S., as one or more interlocking hexagons (see Fig. 1.3).

Fig. 1.3.



Group of six initials surrounding a central cell

Such a structure provides genetic stability and radial symmetry, however, it can only exist as a ring of six surrounding one other initial in the centre, which could be the cell from which the others are ultimately derived. This ring of six initials, if occupied by

cells of two genotypes would allow for the existence of sectors of one half, one third and one sixth, whose stability would be maintained as long as the central cell does not divide. However, division of this central cell anticlinally is possible, and thus a cell from the ring of six would be replaced. Thus the changes in the sizes of sectors observed by Howard and other workers can be explained by the ratio existing in the ring of six initials and the rare, unpredictable changes in the ratio by the irregular participation of the central cell. It has to be remembered that the arguments for six initials are based on existing apices and whether the same arguments can be extended to include de novo formation of apices of adventitious shoots is questionable.

1.2.2. Identification of chimeras

The identification of chimeral shoots can present problems. Ideally a system should be used whereby visual markers allow for early identification of chimeral shoots. However, in the absence of such a system, cytological analysis can sometimes be used. Satina (1940) used measurements of stomatal length as an indication of the ploidy level of the L1 layer of Datura. He found normal diploid plants to have smaller stomata and about twice as many per given area as tetraploid plants. Baker (1943) similarly used stomatal measurements in studying chimeras of Solanum tuberosum. Statistical analysis revealed the stomatal measurements to be highly significant and indicative of the relative size of epidermal cells of the three classes of plants (diploid, tetraploid, octaploid). Sree Ramulu et al., (1975) established the ploidy level of the L1 layer of Lycopersicon

peruvianum plants using stomatal measurements. Hermsen et al., (1970) found that the number of chloroplasts in the stomatal guard cells could be used as a measure of the ploidy level in L1. Working with chimeras produced by colchicine treatment of seeds of Solanum acaule (2n = 48) and Solanum bulbocastanum (2n = 24), Hermsen found that doubling the number of chromosomes resulted in highly significant increases in the number of chloroplasts in guard cells of the stomata.

Information on the L2 layer can be obtained through determination of pollen grain size. Hermsen et al., (1970) utilised the number of germ pores to discriminate haploid and diploid pollen grains, and therefore diploid and tetraploid somatic tissues. The ploidy level of L3 layer is determined directly by counting chromosomes in root-tip cells (Hermsen et al., 1970; Sree Ramulu et al., 1975). It might also be possible to ascertain the ploidy level of the L3 layer by inducing shoot regeneration from root tissue, and determining the ploidy level and cell layer construction of any regenerated shoots. Howard (1964b) and Miedema (1967) developed methods for producing shoots from root tissue, but both methods involved the production of roots from tubers in vivo. Recently, Espinoza et al., (1985) achieved adventitious shoot formation from cultured roots and found that, in some cases, regeneration took place apparently without an intermediate callus phase.

Morphological differences exhibited by the plants can sometimes be exploited to identify chimeral associations. Arisumi (1964) used such differences to distinguish between tetraploid and cytochimeral day-lilies and found that plants tetraploid in L2 layer, regardless

of the ploidy of L1 and L3 layers were easily distinguishable from the controls by larger and heavier petals, larger anthers, thicker styles and thicker flower buds. However, many plants thought to be tetraploid from the appearance of foliage and plant form, were later found to be diploids from microscopic evidence.

Differences in cell size relating to ploidy level can be used in conjunction with other criteria. Satina et al., (1940) used cell size in analysing chimeras of Datura. Stewart (1978) reports on the use of cell and nuclear size in determining ploidy differences. Similarly, Tilney-Bassett (1963) reports "where the layers of a chimera differ according to their chromosome number, the tetraploid cells (compared with diploid cells) are detected by greater nuclear size and an increase in cell size".

1.3. GRAFTING

A graft, although a composite plant, and capable of giving rise to a chimera, is not itself considered to be a chimera. Contact between the tissues of scion and stock occur only at the graft union, and this is the situation throughout the life of the graft. However, a graft provides an example of a composite plant and can demonstrate the reactions which can occur when genetically unlike tissues attempt to live together, a situation encountered in every chimera. Furthermore, the word chimera was introduced into botanical terminology as the result of a grafting operation, and grafting techniques have been used in this study of chimeras.

1.3.1. Grafting of differentiated plant tissues

According to McCully (1983), several different structural events take place during the course of graft development of autografts (the two components of the graft belong to the same plant) and heterografts (the two components of the graft belong to different species). The process of development begins with the formation of a necrotic layer, a complex and dynamic layer composed of remnants of walls and contents of cut cells. In successful grafts, generally much of this layer disappears, as a result of tissue proliferation from either side of the layer. However, in some cases the success of the graft does not appear to depend on removal of the necrotic layer (Camus, 1949; Stoddard et al., 1980).

The next stage involves the expansion of undamaged parenchyma cells, lying immediately under the cut surfaces of both stock and scion, into the graft space. This expansion can occur anywhere along the graft line, but is usually less extensive in closely adhering regions of pith parenchyma. These cells may or may not subsequently divide to form callus, for example, in grafts of grasses, individual cells often expand greatly, without dividing. Callus proliferation is seen to serve three different functions in graft development, namely, rupture of the necrotic layer, provision of cells at the graft interface through which vascular tissue may subsequently redifferentiate, and contribution to the tensile strength of the graft union. In addition to callus proliferation at the cut surface, fairly extensive cellular division takes place away from the cut surface, especially in the cortical and vascular parenchyma regions, the combination of which must bring living cells into con-

tact at the graft interface.

Differentiation of vascular tissue across the graft interface completes the process of graft development, and it has been suggested that it is a critical structural event in the formation of a successful graft (Yeoman et al., 1978). This differentiation of vascular tissue contributes significantly to the mechanical strength of the graft, much of which is due to the presence of xylem elements whose formation is strongly limited by auxin level (Jacobs, 1954; 1956). This auxin level must take the form of a gradient or flux and it is the auxin flux resulting from the release of auxin from severed vascular strands that Sachs (1981) sees as responsible for differentiation of vascular tissue. There was an absence of vascular differentiation in approach grafts of Sedum telephoides where vascular strands were not severed (Moore, 1984). Callus cells adjacent to severed vascular strands in autografts of Solanum pennellii differentiate into a connecting strand of vascular tissue, whereas callus cells adjacent to pith and cortex tissue (that is, not adjacent to severed vascular strands) remain undifferentiated throughout graft development (Moore, 1984).

Close examination of graft development has shown that the first vascular tissues to bridge a graft are derived from direct differentiation of parenchyma and callus cells into xylary elements and sieve tubes (Simon, 1930; Crafts, 1934; Hayward et al., 1939). Correlated with these structural events is an increase in the tensile strength of the graft union, which is a two phase event, beginning with a slow increase in the tensile strength of the union over three to four days followed by a rapid increase over the next seven to eight

days (Roberts et al., 1961; Lyndsay et al., 1974; Moore, 1983).

Initial cohesion between graft partners is thought to be a cellular wound response resulting in the secretion and subsequent polymerization of cell wall precursors into the graft interface. As cut surfaces of plant tissue have adhered to metal foil and wooden sticks, this initial cohesion is considered to be an event not involving mutual cellular recognition. Increasing cohesion occurs due to continued deposition of cell wall materials in callus cells at the graft interface, proliferation of callus tissue and development of fully differentiated wound xylary strands across the graft. Stoddard et al., (1979) observed a marked callus proliferation in successful autografts in pea roots, but observed little cell division occurred in the scions of grafts that were unsuccessful. However, cohesion can occur where there is little or no callus proliferation or vascular differentiation. Stem grafts of tomato and tobacco (McCully, 1983) provide such an example in that an apparently strong union is formed between pith parenchyma cells, but there is little or no callus proliferation or vascular differentiation in this part of graft. Similarly, grafts of monocotyledons resemble pith parenchyma grafts in forming a strong union without callus proliferation or vascular differentiation (Herrero, 1951; Musik, 1958). In vitro grafts between Coleus internodes displayed neither cellular division nor vascular differentiation, yet partners of the graft adhered strongly (Stoddard et al., 1980). Moore (1984), reports on the absence of vascular differentiation in approach grafts of Sedum telephoides indicating that it is not critical to the formation of a successful graft.

Discussion of graft development necessitates consideration of the question of tissue compatibility. There is a need to distinguish between tissue incompatibility and an unsuccessful graft, which is simply due to poor grafting technique. Moore et al., (1981b) see tissue incompatibility as a response which occurs because one or both tissues are physiologically intolerant of the other graft partner, a response, which is, in effect, a rejection response. Yeoman et al., (1976) define compatibility as "the state in which stock and scion contribute to the development of a union, the tensile strength of which is as great as that of the stem above and below the junction".

The early stages of graft development, cell enlargement, callus formation, and at least the first phase of development of cohesive strength are common to both compatible and incompatible grafts. Furthermore, expression of incompatibility can occur at any stage of graft formation. Wound vascular elements may not form, or vascular cambuim activity is not initiated (Deloire et al., 1982) or vascular bridges form but sieve tubes eventually collapse or become lignified (de Stigter, 1959; Schmid et al., 1981). Yeoman et al., (1976) suggest a mechanism whereby graft compatibility-incompatibility is determined by a mutual cellular recognition system. This system operates by modifying a residual cohesion in all grafts, whether compatible or not, to give a higher degree of cohesion in the compatible union. This intensified cohesion is achieved through the formation of a catalytic complex from components released from either side of the graft junction. The catalytic complex initiates a development sequence which eventually determines the success of a graft.

Moore et al., (1981a) argue that the precise involvement of a cellular recognition system in tissue compatibility and incompatibility reactions is difficult to determine and that it may be operative in some plants, but not in others. They admit that some form of cellular recognition might be involved in co-ordinated differentiation of vascular strands across the graft interface, however, they do not rule out the possible role played by a separate morphogenetic determinant. For example, Warren Wilson et al., (1981) suggest that the position and orientation of cambium in grafts between stems and petioles of many dicotyledonous plants is controlled by the ratio of two diffusible morphogens, possibly sucrose and auxin. The authors propose that the cambium subsequently regenerates along a path where the ratio of auxin to sucrose concentration is similar to that of the original cambium. The movement of toxic substance(s) between graft partners has been reported as the cause of tissue incompatibility, for example, between pear scions and quince rootstocks (Gur et al., 1968; Moore, 1983) and between peach scions and almond rootstocks (Gur et al., 1973). A similar claim was made by Moore et al., (1981b) on observing the cell wall suberization and subsequent necrosis of Sedum cells, which occurred after grafting with Solanum. Such a response is ultrastructurally similar to that observed in certain plant cells exposed to phytotoxins (Jones et al., 1974; 1975a; 1975b; Favali et al., 1978; Roebuck et al., 1978).

Stoddard et al., (1979) state that "all living tissues of the plant body may participate in graft callus formation though the degree of activity depends on tissue, species and type of graft". However, recent work by Walker et al., (1985) has suggested that epidermal cells

are genetically incompetent to respond to whatever signals stimulate internal parenchymatous cells to develop wound closures and to establish strong graft unions. The authors carried out approach grafts whereby the internodes of two different shoots were bound together parallel to each other. Five species of flowering plants from different families were studied, but all grafts studied were autografts. The vascular systems were not cut, so that only cortical and epidermal cells were directly involved in the grafts. With all the species studied, the authors found that the presence of an intact, mature epidermis prevented graft formation. However, removal of epidermal tissue from both partners of the graft resulted in a graft union, but, removal of epidermal tissue from one partner merely induced callus proliferation from the cortical cells of that partner, but no adhesion occurred as the intact surface of the other partner maintained its integrity.

The authors suggest that this inability of the intact, mature epidermis to exhibit a graft response reflects both its role as an external tissue and its position within the hierarchy of determined tissues. The inability of an external tissue to form a graft prevents fusion of a spontaneous nature between any contacting tissue, thus maintaining normal, autonomous development. Conversely, it is important for internal tissues to respond to wounding and to engage in cellular processes which will bring about wound repair. With respect to cell determination, this incompetence to graft is associated with the epidermal cells' inability to divide in the periclinal plane and thus form callus, indicating a higher degree of determination in comparison with parenchymatous cells.

1.3.2. *In vitro* grafting

There are few reports of *in vitro* grafting in the literature. Navarro *et al.*, (1975) used shoot-tip grafting *in vitro* to obtain virus-free citrus. *Poncirus trifoliata* (L) Raft x *Citrus sinensis* (L) Osbeck, "Troyer" citrange was used in most experiments as the root-stock cultivar, and a wide range of *Citrus* cultivars were used as a source of shoot-tips. The authors found that neither IAA or BAP influenced the degree of grafting success, however, increasing the sucrose concentration in the medium resulted in an improved grafting success. Similarly, Mampouya, (1983), found that increasing the sucrose concentration in the medium increased grafting success from 40% to 95% in *Citrus* species. Shu-Ching Huang *et al.*, (1980) found that a higher concentration of sucrose in the medium favoured adhesion between stock and scion in *in vitro* "micrografting" of apple shoot-tips onto seedlings of open-pollinated "Golden Delicious" apples. Parkinson *et al.*, (1982) describe the formation of successful autografts of *Lycopersicon esculentum*, *Datura stramonium*, and *Nicandra physaloides*. Formation of these successful grafts was dependent on the application of auxin (IAA) to the apical end of the isolated internode. The addition of kinetin to the culture medium stimulated graft development, but gibberellic acid was inhibitory.

1.3.3. Grafting of dedifferentiated plant tissues

It has been known for some time that plant callus of the same species grown adjacent to each other will fuse (Gautheret, 1945; Joshi *et al.*,1968). However, little attention has been given to the activities of tissues of different species, genera or families in

mixed culture. Explants from trees of willow and poplar, if inoculated adjacent to each other, will fuse after cell proliferation. Conversely, similar explants from elder and maple will not fuse and death of the tissue occurs after several hours (Gautheret, 1945). Callus fusion between different species of fruit trees were investigated by Fujii et al., (1972). They report varying degrees of callus fusion between the species, and microscopic observations revealed boundary layers between the callus explants, the intensity of which appeared to depend on their taxonomic relationship. They do not comment on whether cellular necrosis occurred in any of the callus explants as a result of fusion, though they do state that one callus explant was not seen to inhibit the growth of another. Ball (1969) looked at growth patterns in mixed callus cultures where the two components in the mixture always belonged to different genera or families. In some of the cultures a deeply staining "barrier" layer was observed between the components of the mixed culture. In others, the "barrier" layer was either not present or intermittently so, and Ball suggests that it is being replaced by the derivative cells produced by numerous divisions at the surface of each component.

Moore et al., (1983) used mixed callus cultures of Sedum telephoides callus and Solanum pennellii callus in their study of incompatibility. They wished to ascertain whether the incompatibility response observed between stems of these species required a prior wounding of the tissues, and was the result of a prolonged wound response. Consequently, intact cell masses of both Sedum callus and Solanum callus were placed in contact; care being taken

not to wound the tissues during the process. A necrotic layer made up of cell walls and cytoplasmic remnants was formed at the graft interface and Sedum cells displayed progressive cellular necrosis. These results indicated that prior wounding of tissues is not necessary to elicit an incompatibility response between these two species. Furthermore, these results also infer that the incompatibility factor is present in ground tissue and is not a product of more highly differentiated tissue.

1.3.3.1. Callus induction

The stimulation of cell division and cellular formation is assumed to be either a result of complex interactions between endogenous and exogenous growth regulators, or as a response of the explant to injury of the cells caused by excision, or both conditions acting together. Alternatively, it may result from the removal of the explant from any physical or chemical restraints imposed upon its tissues by the whole plant (Reinert, 1973; Street, 1973). Induction and growth of callus tissue most frequently requires auxins and sometimes a cytokinin is necessary.

Callus cultures can be readily established from a range of Solanum species and from a number of different types of explants; optimum conditions for initiation and growth vary considerably. Callus cultures of potato are usually initiated from either leaf, tuber or stem tissue. High concentrations of auxin, with or without low concentrations of cytokinin in the culture media seem to be favoured by most workers; either NAA or 2,4-D are used in concentrations ranging from 2.0 to 5.0mgl^{-1}. Little emphasis is placed on

the importance of light intensity and temperature in the induction and growth of callus. Lam (1977), however, reported that high auxin and high temperature were favourable to cell proliferation.

1.3.4. Wounding and grafting

Any grafting experiment necessitates wounding of plant tissue, and once wounded, the plant tissue initiates a wound healing process. There are similarities between a wound response and the early events in the formation of a compatible graft, in the sense that both periclinal cellular divisions and callus proliferation occur. Observation of cell enlargement in the wound area prior to division is possibly indicative of auxin presence. However, wounding also results in increased peroxidase activity which can lead to the enzymatic destruction of auxin by peroxidase acting as an IAA-oxidase. In mature tissues, peroxidase is located mainly in the epidermis and in vascular tissues, especially in and near the phloem (Van Fleet, 1959; Andreae et al., 1960). In Helianthus hypocotyls the rate of IAA oxidation was several times faster in epidermal tissue than in epidermis-free tissue (Zenk et al., 1964). Conversely, wounding of tissue also leads to an increase in phenolic substances and it is suggested that these substances may negate the effect of increased peroxidase activity on auxin concentration (Lipetz, 1970). Rosenstock et al., (1978) suggest that the ability of a tissue to react upon wounding with induced mitosis is subject to an aging process, which already begins at an early stage in the development of an organ. There is evidence to suggest that wound reactions may be modulated by the interaction of two or more phytohormones; concentrations of auxin, cytokinin and gibberellin-

like substances have all been shown to increase as a result of wounding.

1.4. REGENERATION

A necessary step in the *in vitro* synthesis of chimeral plants is the regeneration of plants from the chimeral system. In order to achieve the greater recovery of chimeral shoots from a chimeral system, the maximum regeneration potential of that system must be achieved.

1.4.1. Regeneration from callus

Whole plants can be regenerated from callus via an organogenic process involving shoot or root formation, however, the two organogenic processes are loosely connected and it is often necessary to transfer the organ to a second medium for formation of the complementary organ. An organogenic response requires the production of morphogenetically active groups of cells, which later develop into roots or shoots (Reinert, 1962). These groups of cells, often termed primordia or meristematic nodules, may be formed from cells on the surface or in the interior of the callus tissue. This organogenic response was found by Skoog and Miller (1957) to be dependent upon the cytokinin/auxin ratio in the nutrient medium; high ratios of cytokinin in relation to auxin tend to favour shoot formation whereas high auxin ratios favour root formation. However, it would appear that in some plant species, it is the level of endogenous growth regulators which is the critical factor, as shoot and/or root formation occurs without an exogenous supply of growth regulators. In callus cultures of many plant species, root formation

is more frequent than shoot formation.

The morphogenetic capacity of callus is influenced by a number of limiting and interacting factors. Chlorophyll formation in callus is associated with morphogenetic potential and auxin can be inhibitory to chlorophyll formation, for example, some reduction of chlorophyll formation in the presence of 2,4-D was noted in callus cultures of pea, tomato and potato by Hildebrandt et al., (1963). Van Huystee (1977) proposed a permanent inability to synthesise chlorophyll by cells grown on sucrose for prolonged periods of time. This response varies with plant species and there is evidence to suggest that the seemingly permanent breakdown in chlorophyll synthesis can be rectified. Rains et al., (1980), for example, showed that a culture of alfalfa cells which failed to regenerate shoots after three and a half years culture on medium containing three percent sucrose, was induced to form shoots and plantlets through culture on one percent sucrose.

The period of time during which callus tissue has been maintained in culture, commonly influences morphogenetic potential. Street (1979) suggests that callus cultures may consist of morphogenetically competent and non-competent cells or zones. Consequently, failure to isolate a competent cell during subculture may result in its disappearance by the overgrowth of non-competent tissue. Other evidence supports the view that a substance promoting morphogenesis present in freshly isolated explants decreases in vitro (Reinert, 1968), or conversely, a substance inhibiting morphogenesis increases in vitro (Chandler et al., 1982). Rice et al., (1979) propose that mechanisms responsible for activating the genetic

information controlling morphogenesis become lost during subculture. Alternatively, over long periods of culture plant cells can display an altered morphogenetic potential, tissues forming only roots, where previously both shoots and roots had been produced (Negrutiu, 1978).

The first successful establishment of a potato callus tissue culture was reported by Stewart and Caplin in 1951. However, regeneration of plantlets from callus was not achieved until some years later when Lam (1975) reported the induction of embryoid bodies and shoots from potato tuber callus. There have been further reports of plantlet regeneration from potato callus (Skirven et al., 1975; Wang et al., 1975; Roca et al., 1978; Jacobsen, 1981; Ahloowalia, 1982) and from single cell and protoplast culture derived from callus (Behnke, 1976; Lam, 1977; Shepard et al., 1977; Shepard, 1981). The cultural requirements for inducing plant regeneration vary widely. The presence of cytokinin in the culture medium seems to be essential for shoot initiation. BAP has been reported as promoting shoot initiation from callus cultures of potato (Roest et al., 1976; Westcott et al., 1977; Schumann et al., 1980; Webb et al., 1983) and zeatin has been similarly effective (Lam, 1977; Shepard et al., 1977; Schumann et al., 1980). Although there is evidence to suggest that auxin in the culture medium is inhibitory to shoot development, even when carried over from a high concentration in a previous medium (Webb et al., 1983), shoot regeneration from callus has been induced using zeatin in combination with IAA (Shepard et al., 1977; Austin et al., 1982). Several workers have found the addition of GA_3 to the culture medium essential for shoot

development and elongation (Roest et al., 1976; Webb et al., 1983) with the GA_3 apparently promoting shoot development from previously formed shoot meristems. Jarret et al.. (1981) found that although GA_3 was inhibitory to the initiation of shoot meristems from cultured tuber discs, it was absolutely essential for shoot growth and development. Thus, it would seem that the response to growth regulators depends to some extent on the species and cultivar of potato used. Recent work by Wheeler et al., (1984) demonstrated the variability in morphogenetic response from 14 cultivars of Solanum tuberosum. L.

Shepard et al., (1977) found that casamino acids and mineral salts influenced the development of shoot buds critically. In addition, other critical factors were sucrose concentration in the medium, light intensity and temperature during culture. Light intensities in excess of 4000 lux promoted intense greening of protoplast callus, which was positively correlated with the potential of the callus to initiate shoot buds. Low temperatures, (less than $24^{o}C$) were found by Shepard et al., (1977) and Lam (1977) to favour shoot bud initiation.

1.4.2. Regeneration from stem tissue of potato

Reports of shoot regeneration, whether directly or indirectly, that is, via a callus phase, where the original explant have been stem tissue, are limited. The cultural requirements necessary for this organogenic response vary widely. Wang et al., (1975) found that root development occurred more readily than shoot development from callus derived from stem tissue, however, using concentrations of kinetin of $1mgl^{-1}$ to $10mgl^{-1}$ supplemented with a

low concentration of NAA, shoot regeneration could be induced from stem tissue. On the other hand, other evidence favours the presence of zeatin in the regeneration medium in addition to IAA in low concentrations (Schumann et al., 1980; Austin et al., 1982). In addition, BAP in combination with NAA or IAA in the regeneration medium has achieved regeneration of shoots from stem segments of some potato cultivars (Wheeler et al., 1984).

1.4.3. Regeneration from cell layers

The roles of the epidermis and its associated layers in regeneration have been an area of interest over the past decade. Experiments using periclinal chimeras derived from anther cultures of the clone 9-S_1S_3 of Lycopersicon peruvianum, showed that explants devoid of epidermal tissue did not form callus or produced very poor callus after long periods of culture. Furthermore, inner layers were found to be capable of organogenesis in vitro only when cultured with epidermis and it was unclear as to whether the regenerated individuals originated from either L2 or L3 or from both layers (Sree Ramulu et al., 1976). Using stem internodes of periclinal chimeras for chlorophyll deficiency in Nicotiana tabacum, Dulieu (1967 a and b) showed that in vitro regenerated plantlets were of different constitution, originating mainly from either L1 or L2, with a small number of plantlets having their origins in L3 layer.

It has been argued, (Tran Thanh Van et al., 1974) that in culturing thin cell layers (three to eight cells) there should be a relative absence of inter-tissue correlative effects which might occur in large, complex, explants. Furthermore, if it can be

assumed that a reduced volume of explant results in a lower volume of endogenous growth regulators, then the explants should be more sensitive to exogenous substances. Work by Tran Thanh Van (1975) supports this hypothesis in that explants composed of three to six layers of epidermal and sub-epidermal cells excised from floral branches of Nicotiana tabacum are capable of regenerating floral buds, vegetative buds and roots, depending on the concentration of auxin, cytokinin and sucrose in the medium. Slightly less developmental flexibility was achieved from cell layers of Begonia rex, in which epidermal cells could be induced to enter one of several organogenic sequences (vegetative bud or root) or a cytodifferentiative path, i.e. multicellular hair production (Chylah et al., 1975). Similarly, Venverloo (1976) achieved regeneration of shoots and roots from epidermal strips isolated from leaves of Nautilocalyx lyncheii and observed during the process the lesser number of cells involved in the formation of shoot primordia compared to root primordia. In contrast, Bigot (1976) noted only one major response, that of vegative bud production, on cell layers excised from the lower side of leaves of Bryophyllum daigremontianum, even when a wide range of growth regulator treatments were applied.

The studies of Chylah (1974) and Tran Thanh Van et al., (1974b) demonstrate that the epidermal layer of Torenia fournieri can express its bud-forming potential when it is cultured with sub-epidermal tissue. If sub-epidermal tissue is cultured alone, roots are exclusively formed. Similarly, the bud-forming capacity of the epidermal layer of Nicotiana tabacum can only be expressed if it remains in direct or indirect contact with sub-epidermal parenchyma tissue. However, histological analysis of regeneration from

entire stem segments of Nicotiana tabacum reveals that the regenerated organs arise from callus of cambial origin rather than from the epidermis or sub-epidermis.

Walker (1983) discussed these responses of thin cell layers in his study on epidermal cells and their apparent incompetence to adhere, dedifferentiate or divide in random places of division when attempting to form a graft union. He suggests that isolation of these tissues from the mother plant releases them from restraints of morphogenetic factors originating from tissues internal to the epidermis, possibly the vascular tissues. Furthermore, stating the work of Chylah (1974) as an example, he points to the influence of different tissues on the morphogenetic potential on each other, and proposes a form of direct cell communication as a means of co-ordinating cell differentiation in plant organs. Thus, culture of tissues independent of other tissues seems to "liberate" masked regenerative potential in some cases, and to repress the expression of regenerative potential in others. There are certain other factors to consider however, which may affect conclusions made concerning the response of cell layers to in vitro culture. Removal of cell layers necessitates excision, and the explant is therefore subject to wounding and any associated endogenous changes. Furthermore, "simple" cell-layer explants, once cultured in vitro, attain a different, possibly more complex state with respect to their initial organization; it may be that this new "state" is so dissimilar to the original isolated explant that conclusions as to its "released" morphogenetic potential must only be limited.

1.4.4. Effect of wounding on regeneration

The possible effect of wounding on the grafting process has previously been discussed, the question now arises as to the role, if any, wounding plays in the regenerative ability of an explant. Numerous events occur as a result of wounding, the outcome being that most cells in the vicinity of a wound respond by dividing one or more times. The nature of this mitotic stimulus is unclear. The idea of a wound hormone has been suggested, originating from Haberlandt (1921) who demonstrated inhibition of cell division in the vicinity of a wound by washing the wound. Alternatively, changes in endogenous levels and ratios of growth regulators, resulting from wounding, could also induce cell division and these changes would presumably affect regeneration potential.

Takeuchi et al., (1985) looked at the effect of wounding on adventitious bud formation in Torenia stem segments cultured in vitro. Their results suggest that excision of an explant from a mother plant may provoke a series of biochemical events which are closely related to those associated with adventitious bud formation. They found that a high percentage of adventitious buds were formed within a very short distance from the cut ends of the stem segment and that this formation was related to size of segment and presence of cytokinin in the medium. Consequently, it was tentatively suggested that adventitious bud formation in Torenia stem segments requires both application of cytokinin and wounding of the explant.

1.5. GENETIC STABILITY OF PLANT CELLS IN VITRO

Genetic uniformity of plants propagated by tissue culture is an area of much debate. Maximum uniformity is generally associated with the multiplication of non-adventitious axillary shoots, whereas propagation involving an adventitious pathway, especially via a callus phase, is considered to result in varying degrees of heterogeneity or somaclonal variation. Explanations for the difference in genetic stability between organized meristems and proliferating somatic tissues are complex and varied. Evidence from cytological and molecular studies shows that the plant genome can be relatively unstable and subject to various changes in developing somatic tissues. According to D'Amato (1975), polyploidy occurs in the differentiated tissues of 90% of all plant species, thus the nuclear condition of the primary explant will determine, to some extent, the nuclear conditions found in the culture. More than 75% of DNA sequences consist of repetitive DNA which is particularly susceptible to change (Flavell, 1984). Once in culture, other factors become effective. Several workers have demonstrated the importance of the composition of the culture medium in determining the composition of the proliferating cell fraction in a culture (Torrey, 1961, 1967; Matthysse *et al.*, 1967; Bennici *et al.*, 1971). Similarly there is evidence to show that the degree and extent of polyploidy in an *in vitro* culture tends to increase progressively with increasing age of the primary explant or callus or under particular hormonal regimes (Murashige *et al.*, 1967; Bennici *et al.*, 1971; Mehra *et al.*, 1974).

In potato, a wide range of variation in morphological

characters, disease resistance and ploidy levels has been reported among plants regenerated from protoplasts or tissue cultures of several commercial varieties. Sree Ramulu et al., (1985) demonstrated the occurrence of both polyploidization and aneuploidy during in vitro culture of callus and cell suspensions of four genotypes of Solanum tuberosum. Evidence suggested variation was due to in vitro conditions and that those conditions allowed polyploid and aneuploid cells to have a selective mitotic advantage over normal ploidy cells. Similarly, cytological analysis of protoplast-derived regenerants of the cultivars Maris Bard and Fortyfold, has revealed extensive variation in chromosome number (Karp et al., 1982). The authors propose that such variation has resulted from culture conditions used, but also point to the possibility of variation in the original explant. Recent work by Wheeler et al., (1984) reported the regeneration in quantity of plants from various explants of fourteen potato (Solanum tuberosum L) cultivars. Cytological analysis revealed most of the regenerated plants (87%) to contain the euploid number of chromosomes ($2n = 4x = 48$), however, morphological variation in the form of tuber skin, colour, leaf shape and vigour was observed. It has been suggested that somaclonal variation can be due to chromosome rearrangements (Larkin et al., 1981), but demonstration of their occurrence in potato has been very limited (Shepard, 1981). However, plants with structural chromosome changes have been identified among regenerants from protoplasts (Creissen et al., 1985). Alternatively, extranuclear DNA can influence plant phenotype, thus alterations in cytoplasmic genetic elements may contribute to

phenotypic diversity. There is evidence to suggest that mitochondrial DNA variation occurs in protoplast-derived plantlets of potato, however, no chloroplast DNA variation was detected (Kemble et al., 1984).

1.6. PROJECT AIM

The aim of this project was to investigate the possibility of constructing chimeras of potato in vitro, as a means of transferring any beneficial characters that are specifically associated with the epidermal or L1 layer. This aim necessitated the development of chimeral systems whereby induction of regeneration from those systems might result in chimeral shoots. Such a project raises the question of whether adventitious shoots originate from single cells or multicellular groups.

The construction of chimeral systems involved utilisation of various grafting techniques and their associated problems. Several factors have to be considered, for example cell recognition, mechanical mismatch because of cell size, and the wounding effect. Two grafting systems were used, namely mixed callus cultures and epidermal grafts. Regeneration from a potential chimeral system is obviously a key step in the production of chimeral shoots. Regeneration from epidermal grafts necessitated consideration of the positional origin of regenerated shoots and how a wounding response, resulting from the technique of epidermal grafting, would affect regeneration. With mixed callus cultures, it has been inferred in

the literature that the longer the period of culture the more likely it is that different cell lines will mix (Carlson, 1974). However, callus regeneration studies have shown that long term callus cultures show a reluctance to regenerate. Finally, the question which arises from both techniques is that of synchrony. Is it possible for diploid and tetraploid cells to synchronise and co-ordinate the meristematic activity required for shoot regeneration?

CHAPTER 2

MATERIALS AND METHOD

2.1. PLANT MATERIAL

The clonal material used in this study comprised six tetraploid cultivars of *Solanum tuberosum* spp *tuberosum* (Pentland Ivory, Pentland Squire, Majestic, Desiree, Fortyfold and Congo) and four diploid species of *Solanum* (*S. brevidens*, *S. jamesii*, *S. chacoense* and *S. sparsipilum*). Adequate stock cultures of these species and cultivars were maintained in a growth cabinet on a basal semi-solid medium (Section 2.2.) supplemented with 4% sucrose (w/v). These stock cultures were subjected to the following conditions: a temperature of 22 ± 1°C; an irradiance of 85$\mu Mm^{-2}S^{-1}$PAR and a 16h daylength.

2.2. CULTURE MEDIUM

The basal culture medium was that of Murashige and Skoog (Appendix I.I.) supplied by Flow Laboratories, Irvine, Scotland, without sucrose or plant growth regulators. All culture media were adjusted to pH 5.7 before autoclaving at 1.87bar at 120°C for 20 min. and solidified with 0.8% Oxoid No. 3 agar (w/v).

2.3. CULTURE CONDITIONS

Experimental work necessitated investigating a range of temperatures and light intensities which are described in Table 2.1.

TABLE 2.1.

Code	Temperature (^{o}C)	Daylength (h)	Light Intensity ($\mu Mm^{-2}s^{-1}PAR$)
LL/HT(8)	22 ± 1	8	5
LL/LT	22 ± 1	16	20
HL/LT	22 ± 1	16	85
LL/HT	27 ± 1	continuous	27
HL/HT	27 ± 1	continuous	300

2.4. ASEPTIC TECHNIQUE

Sterile polystyrene culture vessels were supplied by Sterilin Ltd., Teddington, Middlesex. Instruments were sterilised by autoclaving at 1.87 bar at 120^{o}C for 20 min. Aluminium box sections (Section 6.1.3.), silicone tubing (Section 6.1.4.) and polytetrafluorethylene tape (Section 6.2.11.) were sealed in a double layer of aluminium foil prior to autoclaving. Where possible, culture media were sterilised at 1.87 bar at 120^{o}C for 15 min. Thermolabile compounds (such as indole-3-acetic acid and gibberellic acid) were filter-sterilised using 0.22μm pore size Millex-GS single-use filter units (Millipore, S.A.). When necessary, plant material was surface-sterilised by immersion in aqueous solutions of sodium hypochlorite containing a wetting agent ("Tween 80" - Atlas Chem. Industries Inc.): a few drops per 100 cm^{3} for 10 to 15 min., and then rinsed thoroughly in sterile, distilled water. All manipulations

involving aseptic plant material and sterile nutrient media were conducted in a laminar airflow cabinet (Microflow, Pathfinder Ltd., Hampshire).

2.5. ESTABLISHMENT OF PLANTLETS IN SOIL

Shoot-tips (5 to 8mm long) were excised from plantlets cultured *in vitro* on semi-solid agar and transferred to tubes containing liquid basal medium (Section 2.2.) supplemented with 4% sucrose (w/v). The shoot-tips were supported on Millcap plugs (supplied by Les Lane Horticultural Products, Bucks., U.K.), which were inserted into the tubes. Culture conditions were as described for plant material (Section 2.1.). Once rooted, plantlets were transferred to pots containing 50% perlite: 50% Levington's Universal compost and grown in a glasshouse at a minimum temperature of 15 ± 1°C with natural lighting. Moisture was conserved through the use of transparent plastic covers placed over the plantlets for a period of two weeks. After this time, the plastic covers were removed and the plants allowed to continue normal growth.

2.6. HISTOLOGICAL TECHNIQUES

A modified version of Johansen's method (1940) was used for histological preparations. Samples were fixed for at least 24h at room temperature (ca 20°C) in FAA (70% ethanol (90%v/v), formalin (5%v/v) and glacial acetic acid (5%v/v)). The samples were dehydrated by passage through an alcohol series as shown in Table 2.2.

TABLE 2.2. Tertiary butyl alcohol (TBA) series

Approximate total % of ethanol	50	70	85	95	100
Distilled water in cm^3	50	30	15	-	-
95% ethanol in cm^3	40	50	50	45	-
Tertiary butyl alcohol in cm^3	10	20	35	55	75
100% ethanol in cm^3	-	-	-	-	25

The samples were left overnight in pure tertiary butyl alcohol. Wax infiltration was achieved by addition of paraffin wax with a melting point of 55°C (BDH Chemicals Ltd., Poole, U.K.) to the pure tertiary butyl alcohol. This mixture of wax and tertiary butyl alcohol was left overnight in an oven set at 78°C for the tertiary butyl alcohol to evaporate. The wax was then poured off and the procedure was repeated a further three times with three changes of wax. When the tertiary butyl alcohol had been completely removed and the samples were totally infiltrated with wax, the samples were solidified. 10μm sections were cut from these wax blocks using a rotary microtome (Reichert, Austria).

The wax ribbons were transferred onto slides and, once dried, the wax was removed by immersion in Histoclear (National Diagnostics Ltd., U.K.) followed by rehydration through an alcohol series (100%, 95%, 70%, 50%, 30% ethanol for 3 min. each) to tap water and stained in toluidine blue (0.05% w/v) for 5 to 20 min. After checking the intensity of the stain, dehydration was achieved by passing the sections through an alcohol series (30%, 50%, 70%, 95%, 100% for

30 secs. each). Slides were left in Histoclear until finally mounted in DPX mountant (BDH Chemicals Ltd., Poole, U.K.), and then viewed under an Olympus BH-Z microscope.

2.7. STOMATAL LENGTH MEASUREMENT

A calibrated Olympus BH-Z microscope was used to determine the length of guard cells. The epidermis was removed from the lower surface of the second and third leaves of shoot-tip derived plantlets (in culture for six weeks), placed on a glass slide and covered with a few drops of distilled water. Guard-cell length was measured using the x40 objective.

2.8. CYTOLOGICAL TECHNIQUES

2.8.1. Quantitative estimation of nuclear-DNA content

A modified version of the technique of Fox (1969) was used. Callus samples were fixed for at least 1h in 100% ethanol : glacial acetic acid (3:1). The material was subsequently collected by centrifugation (500g, 5 min.) and washed 2x in distilled water for a total of 30 min. The material was then hydrolysed in 1M HCl for 10 min. at 60^{o}C in order to remove the RNA from the tissue and liberate the aldehyde groups on the DNA. The suspension was poured over a 4.25cm diameter glass fibre filter (type GF/A) and the cells washed with distilled water under slight vacuum for a period of 1 min. Cells were then collected from the filter with a spatula and resuspended in Feulgen stain (Feulgen and Rossenbeck, 1924). After 45 min. at room temperature the stain was removed and there followed three

washes of 10 min. each in SO_2 water (1MHCl, 10% (w/v) $K_2S_2O_5$ and distilled water in the ratio of 1 : 1 : 20). Finally the cell suspension was centrifuged and the cell material resuspended in 45% acetic acid. One to two drops of this suspension was transferred to a slide, a coverslip added and slight pressure administered to the preparation through several layers of filter paper. The slides were made permanent by sealing the edges with rubber solution (Elan, U.K.). The Feulgen-stained cells were viewed in normal light in a Leitz MPV3 microphotometer in order to identify individual, undamaged nuclei which could then be viewed under short wavelength illumination for fluorescence measurement. The fluorescence was determined using episcopic illumination from a 100W HBO mercury lamp and a Leitz Ploemopac filter block NZ giving an exciting wavelength of 530-560nm with all fluorescence measurements made above 580nm. The fluorescence of nuclei was monitored using x40 objective, individual nuclei being isolated by stopping down the field iris of the episcopic illuminator. Measurements were then taken randomly from several slides prepared from each sample of callus. Background fluorescence was estimated by taking the mean values of areas measured adjacent to the nuclei.

2.8.2. Chromosome preparations

Healthy root tips were collected from freshly rooted plantlets cultured *in vitro* on basal culture medium (Section 2.2.) supplemented with 4% sucrose (w/v). The roots were pretreated with 2mM 8-hydroxyquinoline for 4h at 18°C and then fixed in absolute alcohol : glacial acetic acid (3 : 1) for approximately 48h. The roots were then hydrolysed in 1MHCl for 10 min. at 60°C, washed with distilled water

and finally stained with Feulgen reagent (Appendix I.4.) for 30 min. at 4°C in the dark. Squash preparations were made in 1% acetocarmine in 45% acetic acid (Belling, 1926). Counts were obtained from a minimum of five well-spread cells and at least two separate roots.

2.9. MICROSCOPY/PHOTOGRAPHY

Chromosome preparations and sections were examined with an Olympus BH-Z transmission microscope fitted with an Olympus OM2 camera attachment. Photographs were taken using Kodak Panatomic X film and developed using D76 developer. All photographs of petri dishes were taken with a Zeiss epitechniscope fitted with an Olympus OM2 camera using Kodachrome 64 and Ektochrome 200 film. Non-microscopic photographs were taken with an Olympus OM2 camera using Kodachrome 64 film. For culture examination and all dissecting work, an Olympus VMZ 1x-4x stereo microscope was used.

CHAPTER 3

IMPROVEMENT OF SOURCE MATERIAL

3.1. INTRODUCTION

As all material with the exception of tubers and leaves used in Sections 4.4. and 4.5., was of *in vitro* origin, the following preliminary work was aimed at producing sturdier, larger plantlets, namely in terms of stem diameter, to facilitate grafting techniques to be utilised at a later date. Thus, the effects of sucrose concentration in the culture medium and culture containers on plantlet growth were investigated.

3.2. INFLUENCE OF DIFFERING SUCROSE CONCENTRATIONS ON THE GROWTH OF SOURCE MATERIAL

Shoot-tips were excised from plantlets derived from shoot-tip cultures or axillary bud cultures. Explants were inoculated onto agar contained within a range of containers of different shapes and volumes (Section 3.2.1.). The basal culture medium (Section 2.2.) was supplemented with varying concentrations of sucrose: 3%, 4% and 5% (w/v). Explants were incubated under HL/LT conditions (Section 2.3.). This investigation was carried out using three cultivars of *S. tuberosum* spp *tuberosum* (Fortyfold, Pentland Ivory and Majestic) and two species of *Solanum* (*S. sparsipilum* and *S. chacoense*). For each species or cultivar, six shoot-tips were excised for each treatment.

Table 3.1. shows the response of each species and cultivar to sucrose concentration in the medium in terms of stem thickness and height. 4% sucrose concentration in the medium resulted in sturdier, healthier growth of plantlets than either 3% or 5%.

3.2.1. Influence of container volume and shape on growth of source material

Experimental procedure and cultural conditions were as described in Section 3.2. Containers of differing volume and shape, as outlined in Table 3.1., were used. The 5cm and 9cm petri dishes were sealed with Parafilm (American Can. Co., Greenwich, C.T. 06830) and small holes were pierced in the Parafilm to allow for better aeration. The lids on the universal and specimen containers were left loose to facilitate aeration. Shoot-tips were inoculated singly onto agar in the different containers. For each container and medium combination, the growth of six shoot-tips per species or cultivar was determined.

Plantlet growth was assessed in terms of height of stem, thickness of stem, and whether or not the plantlets looked healthy. It was concluded from the results (Table 3.1.) that plantlets cultured in 9cm petri dishes and 150cm^3 specimen containers were sturdier and healthier than those cultured in the other containers under investigation.

Table 3.1. Key

Media: MS + 3%, 4% or 5% sucrose

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Number of replicates per treatment: 6

Stem diameter: assessed on arbitrary scale of 1 to 3 (where 1 < 1mm; 2 < 2mm; 3 > 2mm)

Volume of media: 10cm^3; 12cm^3; 20cm^3; 35cm^3; 55cm^3

Period of culture: 25 days

TABLE 3.1. Influence of differing sucrose concentrations and different growing containers on development of source material

SPECIES AND CULTIVAR		9CM PETRI DISH VOL $35CM^3$		5CM PETRI DISH VOL $12CM^3$		$60CM^3$ SPECIMEN CONTAINER VOL $20CM^3$		$150CM^3$ SPECIMEN CONTAINER VOL $55CM^3$		$30CM^3$ UNIVERSAL CONTAINER VOL $10CM^3$	
	SUCROSE CONCN. %	AVER. HT. OF STEM (CMS)	AVER. DIAM. OF STEM	AVER. HT. OF STEM (CMS)	AVER. DIAM. OF STEM	AVER. HT. OF STEM (CMS)	AVER. DIAM. OF STEM	AVER. HT. OF STEM (CMS)	AVER. DIAM. OF STEM	AVER. HT. OF STEM (CMS)	AVER. DIAM. OF STEM
PENTLAND IVORY	3	2	3	3.5	3	1.5	1	2.5	3	2.5	2
	4	2.75	3	2.0	2	2.5	1-2	4.5	2	4.0	2
	5	1.5	2	3.0	1-2	1.0	1-2	2.0	3	2.0	2
MAJESTIC	3	1.5	2	1.5	1-2	1.5	2	2.0	1-2	2.0	1-2
	4	2.5	3	2.0	2	3.0	3	2.0	2	3.0	3
	5	1.0	3	2.0	2	2.0	1-2	1.5	2	1.5	1-2
FORTY-FOLD	3	5.5	2	3.5	2	4.0	2	4.0	1	3.0	3
	4	6.0	3	3.0	3	4.0	3	4.5	3	4.0	3
	5	5.0	2	2.0	3	3.0	3	3.5	2	4.0	3
S. SPARSIPILUM	3	2.5	2	3.5	2	3.0	1-2	3.0	3	4.0	3
	4	2.5	3	1.0	2	3.0	2	3.5	2	3.0	2
	5	2.0	1	2.0	1	2.0	1-2	3.0	3	2.5	1
S. CHACOENSE	3	2.5	2	2.5	1-2	2.5	3	3.0	2	3.0	3
	4	2.5	3	1.0	2	2.0	3	2.5	3	2.0	3
	5	1.0	2	1.0	1	1.0	1-2	2.0	2	1.5	1

3.3. DISCUSSION

Sucrose is the most generally used carbohydrate for growth of plant tissue cultures. There are indications that the role of sucrose is a dual one, in that it provides an energy source and is also osmoregulatory (Brown et al., 1978). In the latter role it allows the generation of a turgor pressure which must be established before cell expansion can occur, thus optimising shoot formation and production. The results of the experiment described in Section 3.2. illustrate the effectiveness of sucrose in the culture medium in varying the type of growth exhibited by the shoot-tip cultures. Bearing in mind a sucrose concentration had to be determined whereby optimum growth could be achieved in all species and cultivars investigated, analysis of the results indicated 4% sucrose (w/v) to be that optimum concentration. Work by Constantine (1983) however, on the effect of sucrose concentration on the growth of Prunus domestica cv Victoria shoot cultures found no significant differences in growth (measured by fresh weight and shoot elongation) between concentrations of 2% and 5% sucrose (w/v) in the culture medium. Other studies on the effect of sucrose concentration on growth have tended to concentrate on callus material. For example, Brown et al., (1978) found that tobacco callus grown on a medium containing 3% sucrose (w/v) showed optimum growth and produced the highest number of shoots whereas cultures grown on a medium with lower or higher sucrose levels showed reduced growth rate and capacity to form shoots. Similarly, Murashige et al., (1962) found that a concentration of 3% sucrose (w/v) in the culture medium promoted optimum growth in tobacco callus. As Gamborg et al., (1976) reported, 2% to 4% is the sucrose concentration

(w/v) preferred by most cells: choice within that range would depend to a large extent on the species and cultivar under investigation and the type of culture, and therefore the type of growth required. In this study, the aim was an increased stem diameter which was achieved through using a concentration of 4% sucrose (w/v) in the culture medium.

Many factors are affected by container volume and shape: volume of medium, light availability, gaseous composition and plant support. Experiments with tobacco callus revealed that the yield of tissue obtained was almost directly proportional to the volume of medium utilised and that stoppage of growth occurred due to exhaustion of the entire medium (Murashige et al., 1962). The results described in Section 3.2.1. show that volume of medium alone is not the sole factor determining growth, in that there was seemingly little difference in growth achieved in terms of stem height and diameter between those plantlets cultured in $9cm^3$ petri dishes (containing $35cm^3$ of medium) and $150cm^3$ specimen containers (containing $55cm^3$ of medium). Presumably this response was due to an interaction effect between volume of medium and other factors outlined above. For example, the $9cm^3$ petri dish would favour approximately equal light radiation on all leaves, in that there would be limited shading effect, and although little or no photosynthesis need occur in organs or shoots cultured on media containing sucrose, Hussey et al., (1981) have shown that rate of elongation, thickness of stem, number of nodes and general morphology of shoots of cultivars of Solanum tuberosum ssp. tuberosum were all affected by daylength and light intensity. This experiment has shown therefore, that if an improved growth form is desired, consideration of factors such as light availability and volume of culture medium leading to the selection of an appropriate culture container can be advantageous.

CHAPTER 4

CALLUS PRODUCTION

4.1. INTRODUCTION

Callus production was of interest in this study in so far as an experimental system might be designed allowing genetically distinct cells to proliferate and form a chimeral association, from which chimeral plants might be produced following transfer to an appropriate regenerative medium. Thus, it was important to establish culture conditions that would induce and support callus growth, from both diploid and tetraploid species of potato at a similar rate. Similarity in growth rate was considered a necessity in order to prevent differences leading to dominance of one species by another when cultured together. Preliminary experiments had indicated friability to be an important factor in so far as its effect on the ability of both partners of a mixed callus system to grow together and form a fused mass.

4.2. CALLUS INDUCTION AND PROLIFERATION

Stems of actively growing plantlets derived from shoot-tip cultures were cut into segments, 2 to 3mm in diameter and approximately 1 to 2mm in thickness. The explants were inoculated onto agar with the cut surface nearest the basal end of the plantlet in contact with the agar. Basal culture medium (Section 2.2.) was supplemented with 3% sucrose (w/v) and various combinations of growth regulators as indicated in Table 4.1.

TABLE 4.1.

MEDIA	GROWTH REGULATOR CONCENTRATION (MGL^{-1})			
	2,4-D	NAA	BAP	KIN
1	2	-	-	-
2	2	-	0.2	-
3	3	-	-	0.3
4	-	2	-	-
5	-	3	1	-
6	-	8	-	0.5
7	-	1	-	10.0

Choice of media was based on preliminary experimental work and on results obtained by other workers (Wang et al., 1975; Roest et al., 1970; Lam, 1977a; Wang, 1977). The species and cultivars investigated were as outlined in Section 2.1., except for the inclusion of the two cultivars, Desiree and Congo; for each combination of species, cultivar and medium, 20 explants were cultured. Three light and temperature regimes were used, namely LL/LT, LL/HT and HL/LT (see Section 2.3.). Explants were subcultured onto fresh media every 21 days. Results were assessed after 10, 21, 42 and 63 days of culture.

By day 10, the majority of explants had expanded, the greatest degree of expansion occurring where explants were cultured on media containing the higher levels of growth regulators, for example, kinetin ($10mgl^{-1}$) and NAA ($1mgl^{-1}$). The type of callus produced varied extensively with most explants producing a mixture of callus types. However, by day 21, it appeared that the majority of explants cultured on media containing 2,4-D had produced callus of the more friable type, whereas those explants cultured on media containing NAA had produced callus of the denser, compact type. By day 42, the degree and type of callus production was of the same pattern as that described above (see Table 4.2.). In many explants, the initial, compact, dense callus was being superceded by less compact, more "open" callus and explants cultured on media containing NAA were developing roots. Further assessment revealed that the culture medium supporting similar callus production in both the diploid and tetraploid species contained 2,4-D ($2.0mgl^{-1}$) as the auxin component.

Table 4.2. Key

Media: see Table 4.1. Section 4.2.

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 20$\mu Mm^{-2}s^{-1}$PAR

Number of replicates per treatment: 20

Period of culture: 42 days

%C: % explants producing callus

%FC: % explants producing friable callus

EXC: extent to which explant is covered with callus assessed on an arbitrary scale of 1 to 3 (where 1 < 30%; 2 < 60%; 3 > 60%)

TABLE 4.2. Callus proliferation from stem tissue derived from a range of potato species and cultivars cultured on media containing various concentrations of different growth regulators under LL/LT regime

SPECIES AND CULTIVAR	MEDIUM 1			MEDIUM 2			MEDIUM 3			MEDIUM 4			MEDIUM 5			MEDIUM 6			MEDIUM 7		
	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C
S. brevidens	30	0	1	50	0	1	50	0	1	55	0	1	50	0	1	75	0	1	75	0	1
cv. Majestic	80	15	1	40	0	1	60	0	1	60	0	1	65	0	1	80	20	1	50	0	1
cv. Fortyfold	100	25	1	100	20	1	100	20	1	100	30	3	100	10	2	50	0	1	100	0	2
S. sparsipilum	100	80	2	100	40	2	100	20	2	100	40	2	100	0	2	100	0	1	100	0	2
S. jamesii	100	80	2	100	20	2	100	10	2	35	0	1	55	0	1	55	0	1	65	0	1
cv. Pentland Squire	75	0	1	65	0	2	100	0	2	0	0	0	50	0	1	45	0	1	50	0	1
cv. Pentland Ivory	35	35	2	50	0	1	100	0	2	53	0	1	50	0	1	100	0	1	100	0	1
S. chacoense	100	70	2	50	30	1	80	10	1	40	0	1	45	0	1	100	0	1	100	0	1

Table 4.2. Key

Media: see Table 4.1. Section 4.2.

Temperature: 27 ± 1°C

Light conditions: continuous daylength; 27$\mu Mm^{-2}s^{-1}$PAR

Number of explants per treatment: 20

Period of culture: 42 days

%C: % explants producing callus

%FC: % explants producing friable callus

EXC: extent to which explant is covered with callus assessed on an arbitrary scale of 1 to 3 (where 1 < 30%: 2 < 60%; 3 > 60%)

TABLE 4.2. Callus proliferation from stem tissue derived from a range of potato species and cultivars cultured on media containing various concentrations of different growth regulators under LL/HT regime

SPECIES AND CULTIVAR	MEDIUM 1			MEDIUM 2			MEDIUM 3			MEDIUM 4			MEDIUM 5			MEDIUM 6			MEDIUM 7		
	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C
S. brevidens	100	30	2	55	0	1	50	0	1	70	0	2	50	0	1	95	0	2	100	0	3
cv. Majestic	100	20	2	55	0	1	50	0	1	100	0	2	60	0	1	100	0	2	50	0	1
cv. Fortyfold	100	80	2	100	75	2	100	100	2	100	60	2	100	0	2	100	60	1	100	50	2
S. sparsipilum	75	45	1	100	20	1	100	0	2	100	0	1	100	0	2	100	50	1	40	0	1
S. jamesii	100	60	2	100	0	2	100	0	2	100	0	1	100	0	2	100	50	1	75	25	1
cv. Pentland Squire	100	100	2	100	0	2	100	0	2	100	0	1	100	0	2	100	25	1	100	25	1
cv. Pentland Ivory	100	40	2	100	0	2	100	0	2	100	0	1	100	0	1	100	0	1	55	0	1
S. chacoense	100	0	1	100	0	1	100	0	3	100	0	2	75	0	1	100	0	2	40	0	1

Table 4.2. Key

Media: see Table 4.1. Section 4.2.

Temperature: 22 ± 1°C

Light conditions: 16h daylength, 85μMm^{-2}s^{-1}PAR

Number of replicates per treatment: 20

Period of culture: 42 days

%C: % explants producing callus

%FC: % explants producing friable callus

EXC: extent to which explant is covered with callus assessed on an arbitrary scale of 1 to 3 (where 1 < 30%; 2 < 60%; 3 > 60%)

TABLE 4.2. Callus proliferation from stem tissue derived from a range of potato species and cultivars cultured on media containing various concentrations of different growth regulators under HL/LT regime

SPECIES AND CULTIVAR	MEDIUM 1			MEDIUM 2			MEDIUM 3			MEDIUM 4			MEDIUM 5			MEDIUM 6			MEDIUM 7		
	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C	% C	% FC	EX C
S. brevidens	100	20	2	100	20	2	100	0	2	100	50	2	60	0	1	100	0	2	100	0	2
cv. Majestic	100	100	2	100	75	3	100	30	2	100	80	2	100	0	2	100	80	2	100	20	2
cv. Fortyfold	100	100	2	100	50	2	100	100	3	100	75	2	100	0	2	100	75	2	100	0	2
S. sparsipilum	100	30	2	100	10	2	100	10	2	100	0	2	100	0	2	100	0	2	100	0	1
S. jamesii	100	60	2	75	0	1	100	0	1	100	0	1	100	0	1	50	0	1	85	0	1
cv. Pentland Squire	100	100	2	100	0	2	100	20	1	100	0	2	100	0	1	100	0	2	75	0	1
cv. Pentland Ivory	100	100	2	100	30	2	100	0	2	100	85	2	100	0	2	100	0	3	100	0	2
S. chacoense	100	60	3	25	25	2	100	0	2	100	0	2	100	0	1	100	0	2	100	0	1

4.2.1. Light and temperature influences on callus production

The culture procedure and conditions were as outlined in Section 4.2. After ten days of culture, most explants had developed callus, excluding those cultured under LL/LT (Section 2.3.), however all explants had produced callus after 21 days of culture. A temperature of 22°C seemed most favourable for callus development with all explants, but the extent of friability varied with light intensity; formation of friable callus from the diploid species being favoured under low light ($20\mu Mm^{-2}s^{-1}$PAR) whereas friable callus formation from the tetraploid species was optimal under high light ($85\mu Mm^{-2}s^{-1}$PAR). The results after 42 days of culture are shown in Table 4.2.

4.2.2. Effect of genotype on callus induction and proliferation

The culture procedure and conditions were as outlined in Section 4.2. The volume of callus produced by the tetraploids was favoured under HL/LT conditions (Section 2.3.). All explants from tetraploid plants developed callus at a similar rate with the exception of cv. Fortyfold, where callus growth was markedly slower. The rate of callus production from cv. Fortyfold explants increased when media contained cytokinin in combination with 2,4-D. Fortyfold also exhibited a potential to develop green callus which became more pronounced with culture on media containing 2,4-D. Diploid species with the exception of S. brevidens developed a greater volume of friable callus when cultured on media containing 2,4-D under LL/LT conditions, however, the rate of callus development was slower than with the tetraploid species. Friable callus production from S. brevidens was favoured by medium four which contained NAA ($2.0mgl^{-1}$) under HL/LT conditions.

4.3. EFFECT OF CALLUS CULTURE MEDIA AND EXPLANT ORIGIN ON CALLUS FORMATION AND FUTURE REGENERATION POTENTIAL

The callus induced and maintained on media containing 2,4-D (Section 4.2.) was transferred to a number of media containing different growth regulators in an attempt to induce shoot regeneration (Section 7.12). As Table 7.11 (Section 7.12) shows, shoots were produced from a small number of explants derived from one species only. It was decided to assess the effect the presence of 2,4-D in the callus medium had on the future regeneration potential of that callus, through culturing callus on media lacking in 2,4-D. Therefore media were chosen where the auxin component was not 2,4-D, for although cell division is induced by 2,4-D, there is a tendency also for morphogenesis to be suppressed by 2,4-D (Yeoman et al., 1980). Similarly, media responsible for the induction of shoot regeneration from protoplast callus and long-term callus cultures were also considered (Schumann et al., 1980; Webb et al., 1983; Austin et al., 1983). All media selected, with one exception (ST), were composed of basal culture medium (Section 2.2.) supplemented with 3% sucrose (w/v) and various combinations of growth regulators, as detailed in Table 4.3. For details of medium ST see Appendix I.2.

TABLE 4.3.

CALLUS MEDIA	GROWTH REGULATOR (MGL^{-1})				TEMP. ^{o}C	LIGHT INTENSITY
	NAA	BAP	KINETIN	GA_3		
WO –	0.2	0.2	-	10	22	85
SCH/C	1.0	-	0.2	0.1	22	20
AC	5.0	-	-	-	22	85
JCB	5.0	-	-	-	25	Dark
ST. (Shepard et al, 1977)	0.1	0.5	-	-	22	85

KEY.

Light conditions: 16h daylength; intensity in $\mu Mm^{-2}s^{-1}$PAR.

Thus the aim of this experiment was to assess the media detailed in Table 4.3 with respect to both callus formation and their eventual influence on regeneration potential (Section 7.13). In addition, the effect of explant origin on both callus formation and future regeneration potential was evaluated in that callus was induced from stem tissue as described in Section 4.2 and also from leaf tissue. S. sparsipilum and cvs. Congo and Pentland Ivory of S. tuberosum spp tuberosum were used in this investigation. For each species or cultivar and medium treatment, 40 stem explants and 30 leaf explants were cultured, with the exception of cv. Congo, where due to limited source material, only 20 stem explants and 20 leaf explants were cultured. Subculture of callus to fresh media occurred every three

weeks, with transfer to regeneration media (Section 7.13), after four months of culture on callus induction media.

After one week of culture, stem tissue expressed a greater capacity for callus formation than leaf tissue. Generally callus formation (which was preceded by expansion of the explant) was initiated from the cut ends of the stem section, but as the culture procedure continued, callus formation extended to other areas of the explant. Callus development from leaf sections appeared to be associated with wounded areas, and in some explants, the periphery and mid-rib areas of the leaf. Root formation was favoured in low and high concentrations of NAA, with stem tissue generally showing a greater competence for root regeneration than leaf tissue.

After six weeks of culture callus production was assessed (Tables 4.4.A and 4.4.B). The extent of callus production for the species and cultivars under investigation was in the order: cv.Pentland Ivory > S. sparsipilum > cv. Congo. Callus production was greater from stem tissue than from leaf tissue and the two media achieving callus production resembling that induced by 2,4-D ($2.0 mgl^{-1}$) from both the diploid and tetraploid species were SCH/C and ST. However, of these three media, the medium containing 2,4-D was still superior to the other two media with respect to callus production. The effect of these three media on regeneration potential has been evaluated later (Section 7.13). The effect of the explant from which the callus was derived on regeneration potential was not investigated further due to the apparent reluctance with which leaf tissue produced callus under the conditions tested.

Table 4.4A. Key

Media: see Table 4.3.

Light and temperature conditions: see Table 4.3.

Number of replicates per treatment: 40 (with exception of cv. Congo where number of replicates = 20)

Callus size: 1 = 5 to 10mm in diameter

2 = 10 to 15mm in diameter

3 = 15 to 20mm in diameter

Period of culture: 63 days

TABLE 4.4A. Effect of callus culture media and explant origin (stem tissue) on callus formation

SPECIES AND CULTIVAR	MEDIA	% EXPLANTS FORMING CALLUS	CALLUS SIZE	% EXPLANTS FORMING ROOTS	% EXPLANTS FORMING SHOOTS	TYPE OF CALLUS
PENTLAND IVORY	JCB	100	≥3	60	-	creamy-yellow; friable
CONGO		100	1	55	-	beige-purple; soft
S. SPARSIPILUM		100	2	-	-	creamy-beige; hard
PENTLAND IVORY	AC	100	3	60	-	cream; friable
CONGO		100	1	80	-	beige-purple; gelatinous
S. SPARSIPILUM		100	3	15	-	cream; hard
PENTLAND IVORY	WO	100	2	37	-	cream; gelatinous
CONGO		100	2	50	-	white; hard
S. SPARSIPILUM		100	3	7	-	white; hard
PENTLAND IVORY	SCH/C	100	3	58	-	cream; friable
CONGO		100	1	95	-	beige; gelatinous
S. SPARSIPILUM		100	2	5	-	beige-white; friable and gelatinous
PENTLAND IVORY	ST.	100	3	13	-	cream; friable
CONGO		100	2	30	-	white; very open
S. SPARSIPILUM		100	2	15	-	cream-white; friable

Table 4.4B. Key

Media: see Table 4.3.

Light and temperature conditions: see Table 4.3.

Number of replicates per treatment: 30 (with exception of Congo where number of replicates = 20)

Callus size: 1 = 5 to 10mm in diameter

2 = 10 to 15mm in diameter

3 = 15 to 20mm in diameter

Period of culture = 63 days

TABLE 4.4B. Effect of callus culture media and explant origin (leaf tissue) on callus formation

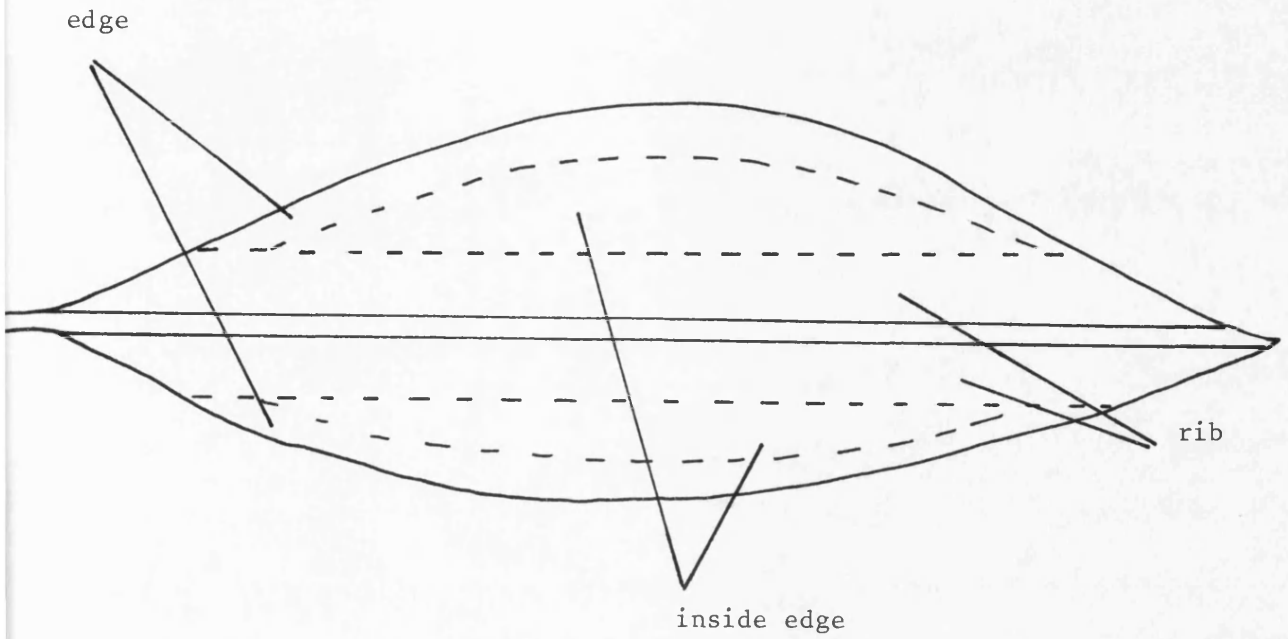
SPECIES AND CULTIVAR	MEDIA	% EXPLANTS FORMING CALLUS	CALLUS SIZE	% EXPLANTS FORMING ROOTS	% EXPLANTS FORMING SHOOTS	TYPE OF CALLUS
PENTLAND IVORY	JCB	100	3	67	-	cream-yellow; friable
CONGO		100	1	25	-	beige-purple; soft
S. SPARSIPILUM		100	1	-	-	cream-beige; hard
PENTLAND IVORY	AC	100	3	87	-	beige; gelatinous
CONGO		100	1	70	-	purple-beige; soft and hard
S. SPARSIPILUM		100	2	63	-	cream; gelatinous
PENTLAND IVORY	WO	37	1	3	-	cream; gelatinous
CONGO		100	1	5	-	white; hard
S. SPARSIPILUM		100	2	-	-	cream-white; open
PENTLAND IVORY	SCH/C	69	1	37	-	beige; hard and compact
CONGO		89	1	60	-	beige-purple; soft and friable
S. SPARSIPILUM		100	2	7	-	cream; hard
PENTLAND IVORY	ST.	100	1	-	-	cream; friable
CONGO		75	1	-	-	white; hard
S. SPARSIPILUM		100	2	-	-	cream; open

4.4. THE INFLUENCE OF CULTURE MEDIUM ON THE PRODUCTION OF PURPLE-PIGMENTED CALLUS FROM CULTIVAR CONGO

Various tissues of the cultivar Congo exhibit a dark purple colour, for example, young leaves and tuber tissue. Consideration was given to the possibility of exploiting this characteristic with the aim of producing a stable purple callus. Such callus would provide a useful marker in indicating the extent to which callus from cv. Congo and another species or cultivar had mixed (Section 5.2).

Leaves of greenhouse-grown Congo cultivar were collected and surface-sterilised in 7% sodium hypochlorite (Section 2.4). Young leaves were selected as they tended to display larger areas of purple pigmentation than the older leaves, however, even in these young leaves, this purple pigmentation was not of a regular occurrence, and was never seen to occur throughout the whole area of the leaf. Sections of leaf, approximately 5mm in diameter, were cut from three different areas of the whole leaf (see Fig. 4.1). These leaf explants were inoculated onto medium with their lower surface in contact with the medium.

Fig. 4.1. Diagram to illustrate areas of leaf used in the production of callus from S. tuberosum ssp tuberosum cv. Congo



The culture media investigated were as described in Section 4.2 (Table 4.1) and the effects of each medium were examined under two different light and temperature regimes, namely LL/LT and HL/LT (Section 2.3). For each of the seven media, a total of 48 leaf explants were cultured under the two different light and temperature regimes. At three-weekly intervals sectors of purple callus were isolated (where possible) and subcultured onto fresh media.

After 21 days of culture, the majority of explants cultured under high light intensity had developed callus. In contrast, only those explants cultured on 2NAA and 2.02,4-D/0.2BAP under low light intensity had produced callus. The type of callus generated varied

from white, compact callus to opaque, loose-grained callus to greenish-brown loose-grained callus. Any purple callus produced was concentrated in small pockets and these tended to be associated with root initials and the mid-rib area. Leaf explants cultured on media containing 3.02,4-D/0.3 kinetin had a greater area of purple callus present than those cultured on any other media (see Table 4.5). Subculture of areas of purple callus onto selected media did not result in any marked further increases in the amount of purple callus present.

TABLE 4.5. Production of purple-pigmented callus from leaves of the cultivar, Congo, using different culture media

MEDIA	LIGHT/TEMP = LL/LT		LIGHT/TEMP = HL/LT	
	% EXPLANTS WITH PURPLE CELLS	EXTENT OF PURPLE PIGMENTATION	% EXPLANTS WITH PURPLE CELLS	EXTENT OF PURPLE PIGMENTATION
1. 2.02,4-D	-	-	17	1
2. 2.02,4-D/0.2BAP	-	-	-	-
3. 3.02,4-D/0.3KIN	-	-	75	2
4. 2.0NAA	42	1	-	-
5. 3.0NAA/1.0BAP	-	-	12	1
6. 8.0NAA/0.5KIN	-	-	-	-
7. 1.0NAA/10.0KIN	17	1	50	<1

Key

Media: see Table 4.1. Section 4.2

Light conditions, LL/LT: 16h daylength; 20 $\mu Mm^{-2}s^{-1}$ PAR

Light conditions, HL/LT: 16h daylength; 85 $\mu Mm^{-2}s^{-1}$ PAR

Temperature: 22 ± 1°C

Number of replicates per treatment: 24

Extent of purple pigmentation: assessed on an arbitrary scale of 1 to 3

Period of culture: 74 days

4.5. PRODUCTION OF PURPLE-PIGMENTED CALLUS FROM TUBERS OF THE CULTIVAR, CONGO

As the internal tissue of Congo tubers is purple in pigmentation throughout the tuber it was decided to investigate the possibility of generating purple callus from these pigmented cells.

Tubers weighing approximately 10g., free from visible, external defects were scrubbed,rinsed with distilled water and then surface-sterilised using 0.5% sodium hypochlorite (Section 2.4). Tubers were cut into halves and 7mm diameter cylinders of tissue, were excised with a sterilised cork borer (No. 5) inserted through the long axis of each tuber. The terminal 2mm portions were discarded and each cylinder was cut into approximately 15 discs of 1mm thickness. The culture procedure and conditions were as detailed in Section 4.4. For each of the seven media a total of 24 explants were cultured under the two different light and temperature regimes.

By day 10, the majority of explants had expanded, however, there had been no production of purple callus. Two callus types were present: white, compact tissue and opaque, gelantinous tissue. The former appeared to originate from the upper surface of the disc, and the latter from around the edges of the disc. By day 30, some purple cells were visible on the uppermost surface of the cultured tissue. These pockets of purple cells were raised, presenting a nodular type of structure, mainly around the perimeter of the discs. The tuber discs revealing the maximum amount of purple-pigmented cells had been cultured on 3.02,4-D/0.3 kinetin under the HL/LT regime (see Table 4.6). As with the leaf explants, (Section 4.4), subculture of these purple-

pigmented sectors onto the medium which seemingly favoured the proliferation of the purple-pigmented cells (3.02,4-D/0.3 kinetin) did not result in any marked further increases in the amount of callus present. Conversely, subculture seemingly encouraged the proliferation of non-pigmented cells, at the expense of the purple-pigmented cells which consequently resulted in a reduction in the number of purple-pigmented cells present.

TABLE 4.6. Production of purple-pigmented callus from tubers discs of the cultivar, Congo, using different culture media

MEDIA	LIGHT/TEMP = LL/LT		LIGHT/TEMP = HL/LT	
	% EXPLANTS WITH PURPLE CELLS	EXTENT OF PURPLE PIGMENTATION	% EXPLANTS WITH PURPLE CELLS	EXTENT OF PURPLE PIGMENTATION
1. 2.02,4-D	-	-	25	2
2. 2.02,4-D/0.2BAP	-	-	33	2
3. 3.02,4-D/0.3KIN	-	-	67	3
4. 2.0NAA	-	-	17	1
5. 3.0NAA/1.0BAP	-	-	-	-
6. 8.0NAA/0.5KIN	50	2	50	1
7. 1.0NAA/10.0KIN	17	1	-	-

Key

Media: see Table 4.1. Section 4.2

Light conditions, LL/LT: 16h daylength; 20 $\mu Mm^{-2}s^{-1}$ PAR.

Light conditions, HL/LT: 16h daylength; 85 $\mu Mm^{-2}s^{-1}$ PAR.

Temperature: 22 ± 1°C

Number of replicates per treatment: 12

Extent of purple pigmentation: assessed on an arbitrary scale of 1 to 3

Period of culture: 68 days

4.6. DISCUSSION

Results from Section 4.2 showed that with the species and cultivars under investigation, media containing 2,4-D alone ($2.0 mgl^{-1}$) gave better results in terms of callus size and type than any other growth regulator or combination of growth regulators. These results are in agreement with those of several workers in that 2,4-D has been shown to induce good callus growth within the potato species. For example, Wang et al., (1975) induced callus from shoot-tips, stems, leaves, root-tips and tuber pieces of potato (Solanum tuberosum L cv. Norin No. I) using a basal culture medium supplemented with 2,4-D ($2.0 mgl^{-1}$). On the other hand, other workers have found other growth regulators to be better at inducing and maintaining callus from various tissues derived from a wide range of different potato species and cultivars (Ahloowalia, 1981; Wheeler et al., 1984). These contradictary results concerning which auxin resulted in maximising callus growth serves to illustrate the variation in response to growth regulators which can occur due to genotypic influence. As reported in Section 4.2.2 genotype would appear to affect callus growth. Results obtained in this study showed that explants of tetraploid origin produced more callus than those of a diploid origin, and that callus production in both cases was favoured by different light intensity. As also noted, variations in callus growth, texture, friability and colour were observed within species.

Light intensity would appear to have some influence on the volume of callus produced and callus friability. Its involvement in the former response is implicated in the results reported in Section 4.2.2, where there would seem to be some interaction with the genotype

of the explant resulting in observable differences in callus growth between diploid and tetraploid species. The effect of light intensity on callus friability was noted and reported in Section 4.2.1. As with callus growth the effect would appear to be the result of some interaction with the genotype of the explant. With the diploid species, a lower light intensity resulted in the formation of a greater volume of more friable callus than the higher light intensity, whereas when the explant was derived from a tetraploid species, the reverse was true. Little evidence of this effect on potato could be found in the literature, however, Osifo (1983) observed that callus formation and growth of tissue derived from Solanum brevidens was highest in full light and least in the dark.

Anthocyanins are produced by tissue and cell cultures of several plants. Blakeley et al., (1961) observed that anthocyanin accumulation in cell cultures of Haplopappus gracilis depended on the auxin concentration of a medium containing coconut water as an essential ingredient. Similarly, Constabel et al., (1970) noted that the anthocyanin level in cell cultures of Haplopappus gracilis increased when the NAA concentration was 10^{-7}M ($0.019 mgl^{-1}$) but not at 10^{-6} ($0.19 mgl^{-1}$). At 10^{-7}M NAA, the cultures remained pigmented and anthocyanin accumulation could be restored after a temporary loss of pigmentation due to an earlier, higher auxin concentration. Optimum pigment accumulation occurred with concentrations of 4.5×10^{-6}M 2,4-D ($1.0 mgl^{-1}$). Davies (1972) in work with Paul's Scarlet Rose (Rosa species) suggested that initiation of pigment synthesis is insensitive to auxin concentration of the medium, but that high levels of 2,4-D ($11 mgl^{-1}$) increased net anthocyanin accumulation by prolonging the period over

which it is synthesised.

In this study, initial observations inferred the existence of a relationship between growth regulators and anthocyanin expression, with explants cultured on media containing 2,4-D ($3.0 mgl^{-1}$) and kinetin ($0.3 mgl^{-1}$) showing higher levels of anthocyanin than explants cultured on other media. However, attempts to select conditions whereby anthocyanin initiation and production could be controlled, failed; repeated subculture led to the establishment of callus cultures in which the dominant cell type was non-pigmented. Control of anthocyanin production for use as a marker would require more extensive study.

CHAPTER 5

GRAFTING OF DEDIFFERENTIATED TISSUE

5.1. INTRODUCTION

The aim of this investigation was to establish the extent to which callus derived from Solanum species of different ploidy levels might grow together, with the eventual intermingling of cells. Subsequent provision of conditions favouring regeneration might then result in the production of chimeral shoots.

5.2. MIXED CALLUS CULTURES

As a result of experiments on callus induction and proliferation (Sections 4.2 and 4.3), callus from both diploid and tetraploid species, and of the required colour and friability, was selected for the formation of mixed callus cultures (Plate 5.1A). The colour of callus was considered as a visual factor which could be used to determine the viability of the callus with respect to future growth and organogenesis. For this purpose, callus exhibiting any signs of brown colouration was not selected and preference was given to callus of green to yellow colouration. Callus of a friable texture was selected in preference to very dense, or very open and loose callus, for as already discussed (Section 4.1), friability was considered to be a factor which could influence the ability of two different callus cultures to grow together. With these factors in mind, callus derived from the following species and cultivars were used to form mixed callus cultures: S. tuberosum Ssp tuberosum cv. Pentland Squire, cv. Pentland Ivory, cv. Majestic, S. sparsipilum and S. chacoense.

Isolated pieces of callus from differing species were placed in contact with each other in a number of different ways:

SS: pieces of callus of similar size were placed side by side

OT: pieces of callus of similar size were placed on top of each other

MC: pieces of callus of similar size were chopped and randomly mixed into a single mass on the surface of the medium

The species combination within a mixed callus culture was as detailed in Table 5.2. Controls for each of the treatments listed, consisted of mixtures of callus from the same genotype. Mixed callus cultures were incubated on the same medium as that responsible for their induction and proliferation, and under the same light and temperature conditions (Table 5.1).

TABLE 5.1

Culture Medium	Temperature (^{o})	Light Conditions $\mu Mm^{-2}s^{-1}$ PAR
2,4-D (Section 4.2)	22 ± 1^{o}C	20 + 85
ST (Section 4.3)	22 ± 1^{o}C	85
SCH/C (Section 4.3)	22 ± 1^{o}C	20

Light conditions in all cases were based on a 16h daylength. Cultures were transferred to fresh media every three to four weeks and assessed in a number of different ways: the morphology of mixed callus cultures; quantitative analysis (that is, the effect of mixing callus of different species on the growth of callus); extent of intermingling of the cells (determined using quantitative nuclear-DNA estimations). Cultures were also transferred to regeneration media after four months.

Although callus pieces had been healthy prior to mixing, after two months of culture there was some indication in a proportion of explants of browning or necrosis. This took place mainly in the two treatments, OT and MC; with the former system necrosis only developed within the tissue of the upper partner (Plate 5.1B). Culture of callus side by side (SS) appeared to result in one of three responses:

1. both partners of the mixed culture seemingly tolerant of each other, growing to produce a culture where the original line of contact was sometimes indistinguishable from the overall mass of tissue (Plates 5.1C and 5.1D).
2. one partner of the mixed culture (usually the tetraploid partner) exhibiting necrosis (Plate 5.1E)
3. as two, but with a tendency for the partner exhibiting necrosis to be swamped by the growth of the other partner of the mixed culture (Plate 5.1F).

As a general rule, however, the first response as described above occurred most frequently, and transfer of the mixed callus system, either during subculture or to a regeneration medium, did not result in the two pieces breaking away from each other.

After four and six months of culture, it was the mixed callus systems initiated through side by side contact which, in the main remained healthy and in a state of active growth. Of the species combinations investigated, S. sparsipilum, with S. tuberosum ssp tuberosum cv. Pentland Ivory proved to be more successful than other combinations in producing an apparently fused mass (Table 5.2).

Of the media utilised in this experiment, the medium containing 2,4-D ($2.0 mgl^{-1}$) appeared to be optimum in inducing callus of the required nature at a similar rate from both partners. Where necrosis was observed after two months of culture, further culture had resulted in what appeared to be complete cellular death of one partner, and in all cases this was the tetraploid partner. In a limited number of explants, however, the browning initially observed had remained confined to small areas and had not increased with culture.

Examination of the cultures set up as controls revealed that fusion of the separate callus pieces had occurred to a greater degree than with the mixed callus systems (Table 5.2). Cellular necrosis was also apparent and as with the mixed callus systems had taken place less when the callus pieces had been placed side by side (SS) rather than either the OT or MC system. Furthermore, the necrotic response was observed more often when the tissue had been derived from a tetraploid species rather than from a diploid species. With the majority of explants, when fusion between callus partners did not occur, necrosis was observed. It was not possible to determine whether fusion had taken place when callus had been mixed using the system MC.

Sections through mixed callus systems were taken and compared with sections through callus of single species, the aim being to assess the extent of mixing of callus from the different species when in mixed culture. However, it was decided that any evidence gained through histological examination was inconclusive due to the varied nature of callus cells in culture.

Table 5.2. Key

Media and light intensity:	see Table 5.1, Section 5.2.
Light conditions:	16h daylength
Temperature:	22 ± 1°C
Number of replicates per treatment:	15-20
Period of culture:	6 months
OT/SS/MC:	see Section 5.2.

TABLE 5.2. Response of callus derived from potato species of different ploidy to co-culture

Species Combination	System of Mixing	% explants showing successful fusion	% explants with necrotic areas
S. sparsipilum + cv. Pentland Ivory	OT	23	77
S. sparsipilum + cv. Pentland Ivory	SS	61	22
S. sparsipilum + cv. Pentland Ivory	MC	-	70
S. sparsipilum + cv. Pentland Squire	OT	22	78
S. sparsipilum + cv. Pentland Squire	SS	53	47
S. sparsipilum + cv. Pentland Squire	MC	-	65
S. chacoense + cv. Majestic	OT	18	78
S. chacoense + cv. Majestic	SS	29	59
S. chacoense + cv. Majestic	MC	-	71

Table 5.2. (CONTINUED). Key

Media and light intensity:	Table 5.1, Section 5.2.
Light conditions:	16h daylength
Temperature:	22 ± 1°C
Number of replicates per treatment:	15-20
Period of culture:	6 months
OT/SS/MC:	Section 5.2.

TABLE 5.2. (CONTINUED). Response of callus derived from potato species of the same ploidy to co-culture

Control species	System of Mixing	% explants showing successful fusion	% explants with necrotic areas
cv. Pentland Ivory	OT	58	42
cv. Pentland Ivory	SS	68	32
cv. Pentland Ivory	MC	-	78
cv. Pentland Squire	OT	50	39
cv. Pentland Squire	SS	64	21
cv. Pentland Squire	MC	-	76
S. chacoense	OT	61	27
S. chacoense	SS	70	25
S. chacoense	MC	-	50
S. sparsipilum	OT	65	20
S. sparsipilum	SS	80	20
S. sparsipilum	MC	-	45
cv. Majestic	OT	55	35
cv. Majestic	SS	60	25
cv. Majestic	MC	-	79

Plate 5.1. Callus induced from stem tissue of potato through culture on medium containing 2,4-D

A. Six month old friable callus derived from stem tissue of S. sparsipilum x 1

B. Mixed callus culture of four months consisting of S. sparsipilum as the upper callus partner and S. tuberosum ssp tuberosum cv. Pentland Squire as the callus in contact with the medium; necrosis had developed within the callus of S. sparsipilum. (A) x 1

C. Mixed callus culture of six months consisting of callus derived from S. sparsipilum and S. tuberosum ssp tuberosum cv. Pentland Ivory placed side by side on the medium; the original line of contact is now indistinguishable x 1

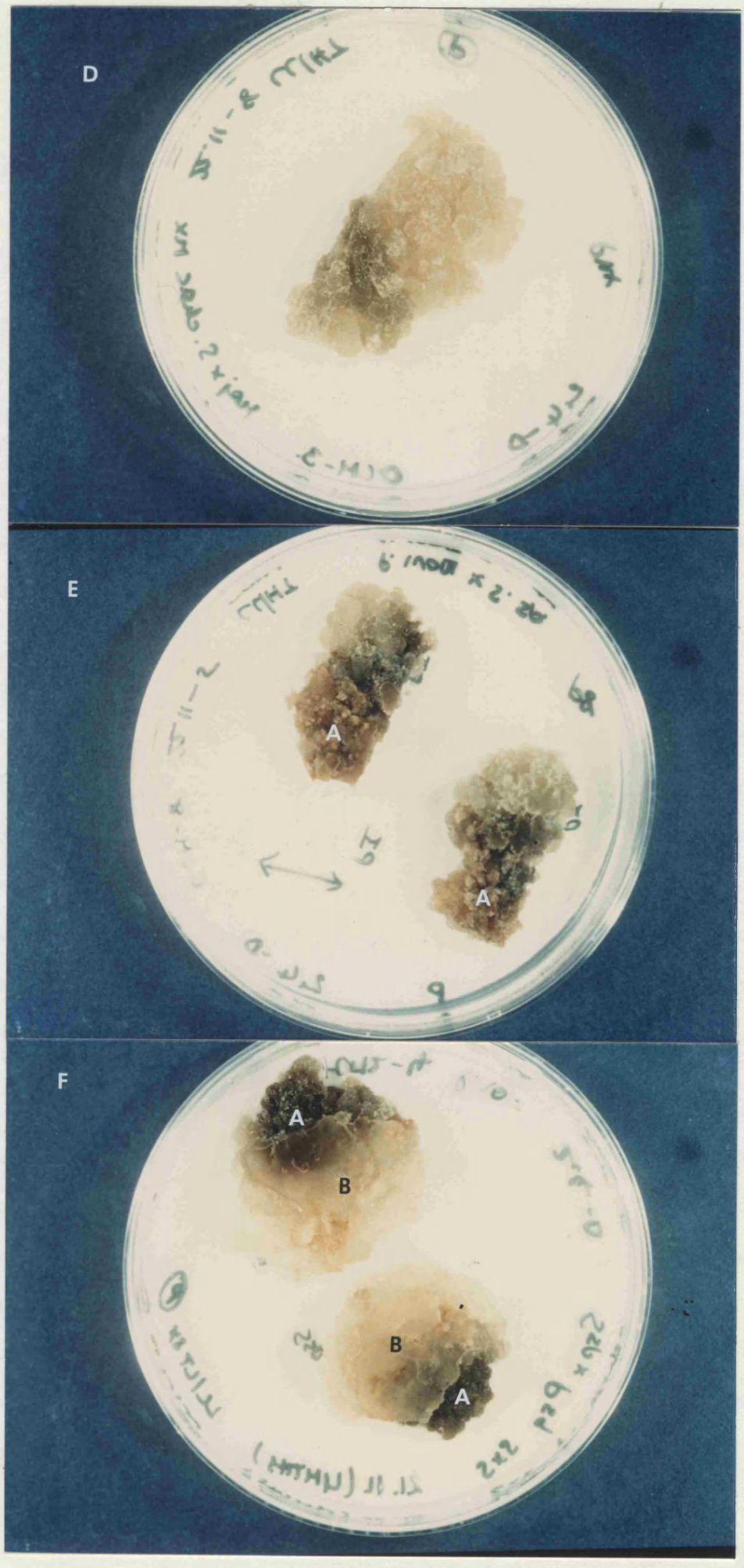
A
B
A
C

Plate 5.1. Callus induced from stem tissue of potato through culture on medium containing 2,4-D

D. Mixed callus culture of four months consisting of callus derived from S. chacoense and S. tuberosum ssp tuberosum cv. Majestic placed side by side on the medium; the original line of contact is now indistinguishable x 1

E. Mixed callus culture of six months consisting of callus derived from S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory placed side by side on the medium; cv. Pentland Ivory (A) is exhibiting necrosis x 1

F. Mixed callus culture of four months consisting of callus derived from S. sparsipilum and S. tuberosum ssp tuberosum cv. Pentland Squire placed side by side on the medium; cv. Pentland Squire (A) is exhibiting necrosis and is being dominated by the growth of S. sparsipilum (B)


D
E
A
A
F
A
B
B
A

5.3. GROWTH OF MIXED CALLUS CULTURES

The aim of this experiment was to ascertain the effect that the co-culture of different species of callus had on the rate of growth of the individual species.

Mixed callus systems were established using callus which had developed on 2,4-D media (Section 4.3) and which was induced from stem tissue derived from S. sparsipilum and S. tuberosum ssp tuberosum cv. Pentland Ivory (Section 4.2). Culture procedure and conditions were as detailed in Section 5.2 except that the only mixed callus system investigated was that involving the placing of pieces of callus side by side (SS), as results described in Section 5.2 had indicated that this method was superior in terms of balanced development of the two calluses. Callus was weighed prior to mixing and was reweighed after one and two months of culture. Within this period of time the administration of gentle pressure succeeded in separating the two components of the mixed callus culture for weighing purposes. Controls were set up involving pieces of callus of one species grown under the same conditions as the mixed callus cultures.

The results of this experiment which are shown in Table 5.3 (for actual numbers see Appendix II..IA and B) indicate that of the two species, it is the tetraploid species whose growth is inhibited as a result of being cultured with callus of another Solanum species of different ploidy level. For example, when callus cv. Pentland Ivory was cultured in isolation for a period of two months, there was an increase in fresh weight of 2.003g., however, when co-cultured with S. sparsipilum for the same periods of time, cv. Pentland Ivory

achieved an increase in fresh weight of 1.412g., a 30% decrease in growth. Although S. sparsipilum did not realise the same increase in fresh weight when co-cultured with cv. Pentland Ivory, as it did when cultured in isolation, the reduction in growth over a period of culture of two months, was only 11%. Hence, it would appear that co-culture affected the growth of S. sparsipilum less than cv. Pentland Ivory.

TABLE 5.3. Effect on growth (measured in weight increase) of callus when co-cultured with callus derived from a different potato species

Species and cultivar	Cultural conditions	Duration of culture (months)	Fresh weight increase (g)
S. sparsipilum	single	1	0.49
S. sparsipilum	mixed	1	0.453
S. sparsipilum	single	2	1.095
S. sparsipilum	mixed	2	0.92
cv. Pentland Ivory	single	1	1.076
cv. Pentland Ivory	mixed	1	0.78
cv. Pentland Ivory	single	2	2.003
cv. Pentland Ivory	mixed	2	1.412

Key

Media: 2,4-D (Section 4.2)

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Temperature: 22 ± 1^{o}C

Number of replicates per treatment: 15-20

5.4. NUCLEAR-DNA MEASUREMENTS

This experiment was undertaken in the hope that examination of nuclear-DNA content through a mixed callus culture would give an indication of the extent of intermingling of the cells within the culture, and demonstrate if there was any tendency for cells of one ploidy level to dominate those of a different ploidy level. However, any analysis of nuclear-DNA contents within a callus system must acknowledge that plant cells grown in vitro, whether as callus or suspension do not usually demonstrate a stable chromosome complement (Bayliss, 1980; Karp et al., 1985). Cell cultures of potato are no exception to this in that changes in chromosome number and/or nuclear-DNA content of callus cells of haploid, diploid and tetraploid plants have been described by Jacobsen (1981), Khivilkovskaya (1982), Jacobsen et al., (1983), Sree Ramulu et al., (1984) and Tempelaar et al., (1985). Thus nuclear-DNA analysis would also provide some indication as to the stability of the callus cultures used in the grafting systems, a factor worth considering in any study involved with the regeneration and characterization of a specific genotype.

Feulgen-stained preparations were made as described in Section 2.8.1. from various callus systems cultured on 2,4-D ($2.0 mgl^{-1}$) medium (Section 5.2) for differing periods of time (for details see Table 5.4). All callus systems were sampled three to four days after sub-culturing onto fresh medium. With the exception of callus system seven (cv. Pentland Ivory + S. sparsipilum - mixed (SS)), portions of callus systems were randomly isolated (four from each callus explant) and slides prepared from which 100 to 150 nuclei were measured. With callus system seven, samples of tissue were excised as shown in Fig. 5.1.

TABLE 5.4.

Species/cultivar	Type of callus system	Duration of culture (months)	Number of callus systems investigated
1. cv. Pentland Ivory	single	3	6
2. cv. Pentland Ivory	single	13	6
3. S. sparsipilum	single	3	6
4. S. sparsipilum	single	13	6
5. cv. Pentland Ivory + S. sparsipilum	mixed (OT)	9	6
6. cv. Pentland Ivory + S. sparsipilum	mixed (MC)	9	6
7. cv. Pentland Ivory + S. sparsipilum	mixed (SS)	4	2
8. S. sparsipilum + cv. Pentland Ivory	mixed (OT)	9	6

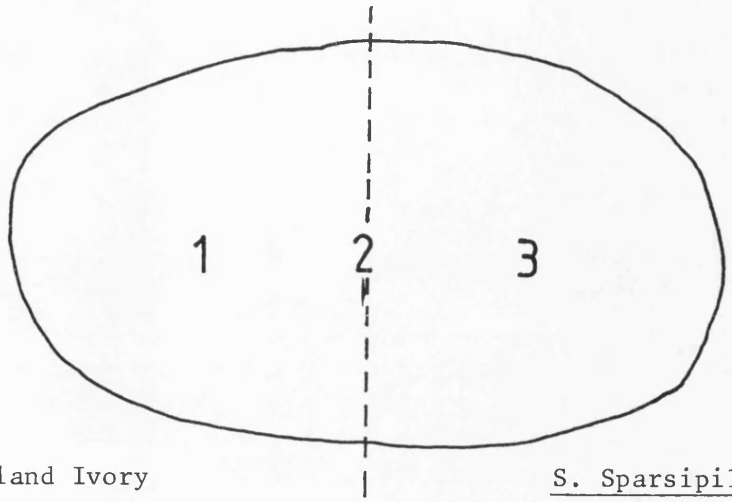
Key

OT: pieces of callus of similar size were placed on top of each other

SS: pieces of callus of similar size were placed side by side

MC: pieces of callus of similar size were cut up and randomly mixed into a single mass on the surface of the medium

Fig. 5.1.



cv. Pentland Ivory S. Sparsipilum

The initial point of contact between two callus samples is indicated by the dotted line, and areas of tissue sampled are represented by numbers one, two and three. All nuclear-DNA content values were grouped and plotted in histogram form (Figs. 5.2)(see Appendix II.2 for actual numbers).

The DNA contents of callus cells derived from diploid tissue after three months culture on 2,4-D medium are represented by Fig. 5.2A. Nuclear-DNA values between ten and 50 units are shown by the frequency distribution with a predominance of cells showing values between ten and 30 units. A similar spectrum of DNA values was obtained on examining callus cells derived from the same diploid tissue, but after a culture period of 13 months duration (Fig. 5.2B). This was interpreted as indicating that the general condition of callus derived from the diploid S. sparsipilum was relatively stable (at least in terms of ploidy level) after 13 months of culture on medium containing 2,4-D ($2.0 mgl^{-1}$). For both callus systems sampled there appears to be only one population of nuclear DNA.

The DNA contents of callus cells drived from tetraploid tissue after three months of culture on 2,4-D medium are represented by Fig. 5.2C. A range of DNA values between 30 and 90 units was detected with a greater proportion of the nuclei (77%) having DNA values between 40 and 60 units. The frequency distribution of nuclear-DNA values obtained from callus cells of tetraploid origin after a culture period of 13 months (Fig. 5.2D) was not unlike that obtained from callus cells in culture for three months, in that the DNA values were within the same range and the same peaks were observed.

A greater spread of DNA values was revealed by the mixed callus systems five and eight (Table 5.4), as shown by Figs. 5.2E and 5.2F. A comparison of these histograms with those relating to diploid tissue (Figs. 5.2A and B) and tetraploid tissue (Figs. 5.2C and D) revealed a tendency for the DNA values to be concentrated within the ten to 50 units range, thus bearing more resemblance to the frequency distribution obtained for diploid tissue rather than tetraploid tissue. However, as shown by Fig. 5.2E, DNA values outside this range were detected in mixed callus system number five.

The nuclear-DNA contents of callus cells from the mixed callus system number six (Table 5.4) are represented by Fig. 5.2G. As Fig. 5.2G shows, the range of DNA values obtained from examination of this callus system has increased and the major peaks detected show little resemblance to those associated with diploid and tetraploid tissue. The scattering of nuclear-DNA values obtained from examination of this system was generally broader with less pronounced

grouping of DNA values than previously observed. The data obtained from DNA analysis of mixed callus system number seven (Table 5.4) is summarised in Figs. 5.2H, 5.2I and 5.2J. As these histograms show, no obvious peaks in nuclear DNA were detected in this system, instead a broad scattering of DNA values between ten and 90 units were revealed.

Figs. 5.2. Key

Histograms are representative of the DNA content of nuclei from callus cells cultured on 2,4-D medium ($2.0 mgl^{-1}$) for varying periods of time.

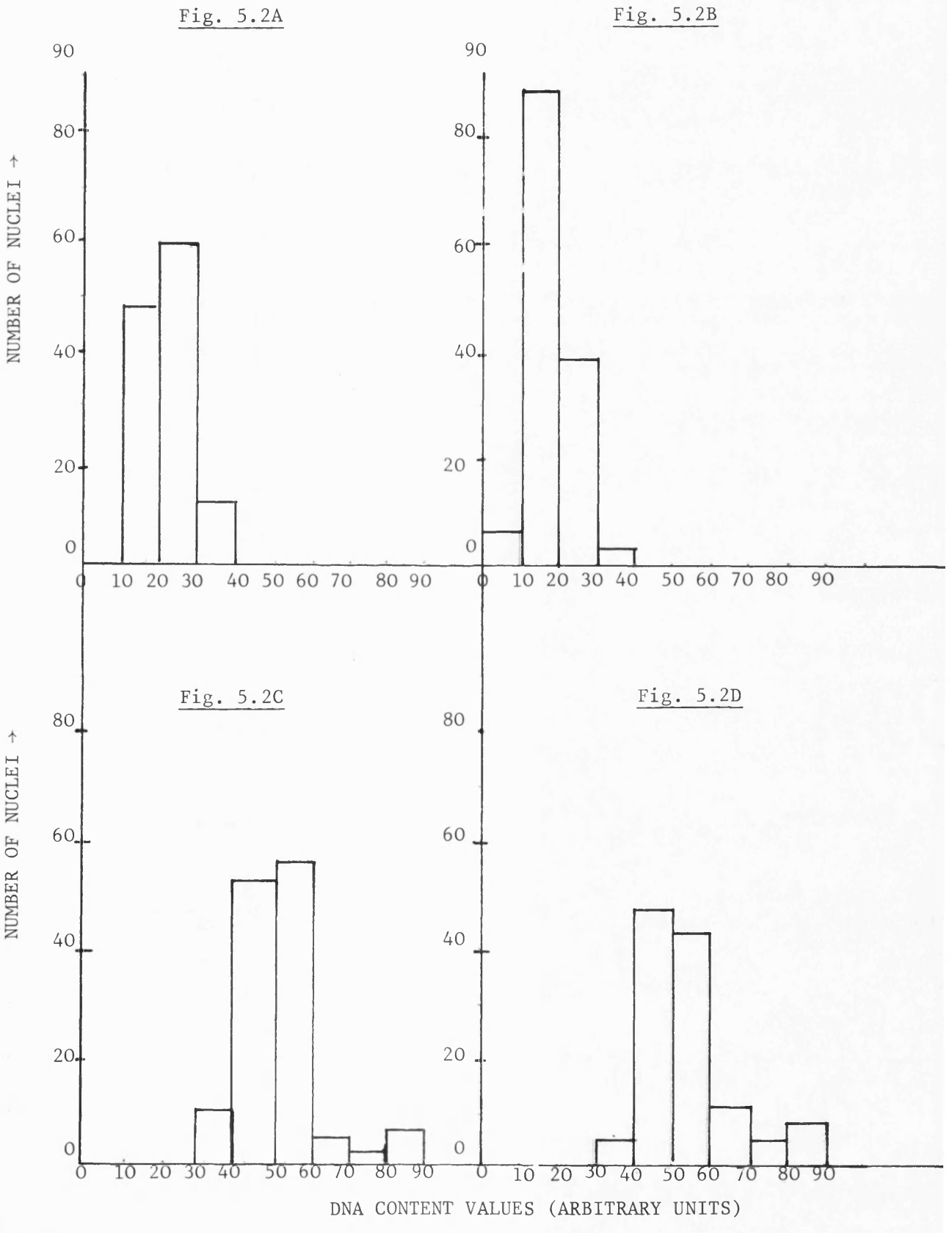
Fig. 5.2A. *S. sparsipilum* cultured for three months.

Fig. 5.2B. *S. sparsipilum* cultured for thirteen months.

Fig. 5.2C. *S. tuberosum* ssp *tuberosum* cv. Pentland Ivory cultured for three months.

Fig. 5.2D. cv. Pentland Ivory cultured for thirteen months.

Figs. 5.2. Histograms of nuclear-DNA content from single callus cultures of S. tuberosum cv. Pentland Ivory and S. sparsipilum



Key (Figs. 5.2E to 5.2J inclusive)

Histograms are representative of the DNA content of nuclei from callus cells cultured on 2,4-D medium ($2.0 mgl^{-1}$) for varying periods of time.

Fig. 5.2E. cv. Pentland Ivory and S. sparsipilum co-cultured (OT) for nine months with cv. Pentland Ivory uppermost.

Fig. 5.2F. as 5.2E but with S. sparsipilum uppermost.

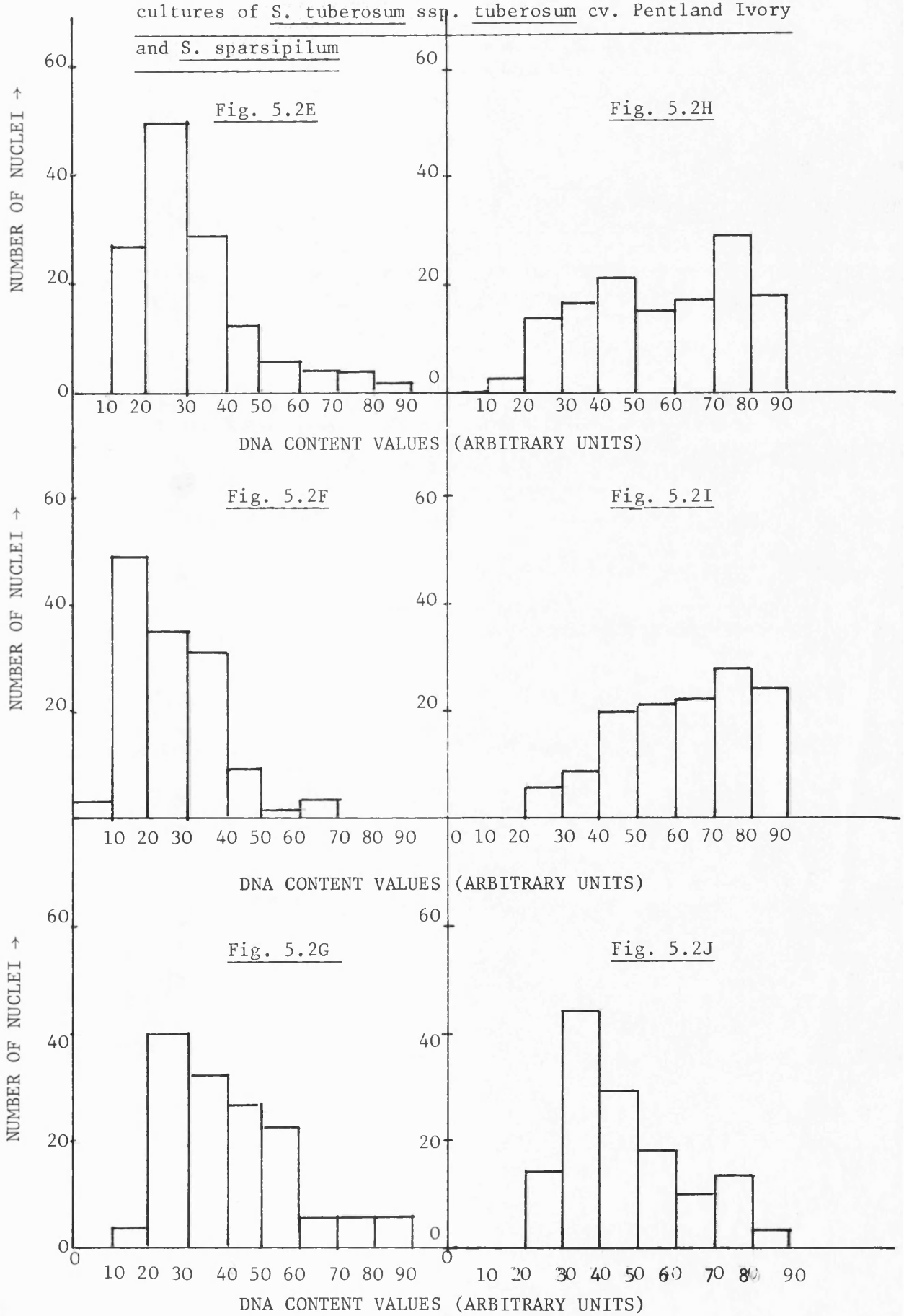
Fig. 5.2G. cv. Pentland Ivory + S. sparsipilum co-cultured (MC) for nine months.

Fig. 5.2H. a sample of callus as indicated by point I (see Fig. 5.1) from mixed callus of cv. Pentland Ivory + S. sparsipilum co-cultured (SS) for four months.

Fig. 5.2J. As Fig. 5.2H but callus sampled at point 2 (Fig. 5.1).

Fig. 5.2J. As Fig. 5.2H but callus sampled at point 3 (Fig. 5.1).

Fig. 5.2. Histograms of nuclear-DNA content from mixed callus cultures of S. tuberosum ssp. tuberosum cv. Pentland Ivory and S. sparsipilum



5.5. DISCUSSION

As discussed in Section 1.3.2., the ability of plant callus of the same species to fuse, if given suitable conditions, has been known for some time. Less study has been made of the response of callus from different species in a mixed callus system. Of the three systems of mixing callus used in this study, that of side by side (SS) proved to be the most successful, in that the other two systems showed more visible signs of necrosis. As no ultra-structural studies were carried out on tissues involved in these mixed cultures, any comments relating to necrosis in this study refer to external signs such as browning and blackening of the tissue, excessive wetness and termination of growth.

Cellular necrosis is a response observed by many workers when incompatible tissues become associated within a grafting system (Copes, 1969; Fletcher, 1964; Moore *et al.*, (1981b). Moore *et al.*, (1983) observed necrotic symptoms when grafting *Sedum* callus with *Solanum* callus. As reported in Section 1.3.2., the *Sedum* cells experienced progressive cellular necrosis; *Solanum*, however, exhibited only a mild wound-like response on grafting, suggesting that some factor associated with *Sedum* cells affects *Solanum* callus cells, but is only mildly toxic or is largely detoxified and/or excluded by the *Solanum* cells. The same authors noted that the cellular responses were similar to those of non-grafted cells exposed to phytotoxins and consequently suggested that incompatibility was linked to a diffusible chemical. Thus the necrosis observed within certain tissues in this study could be an incompatibility reaction resulting from the presence of a substance which one partner in the graft is

adversely sensitive to; in this study the tetraploid species would appear to be the most sensitive partner.(Section 5.2, Table 5.2).

Alternatively there is the question of wound response and Moore et al., (1983) drew attention to the similarity that exists both structurally and biochemically between the incompatibility response of Sedum and Solanum and wound responses in non-grafting systems. An adverse response to wounding rather than an adverse response to the presence of a toxin could explain why grafted callus of the same species experienced necrosis and furthermore could account for why the system involving the chopping of callus (MC) was more susceptible to necrosis than the other systems used, as the production of this system would inflict maximum damage to the cells. Progressive cellular necrosis would be the result of an inability on the part of the tissue to control the wounding response, and where this was more apparent with the tetraploid species than the diploid species could reflect the sensitivity of the former to wounding. When necrosis was observed during the initial stages of culture but did not increase with further culture, this could be indicative of the tissue stabilising, having arrested an initial wound response.

A factor specific to the mixed callus system involving pieces of callus of similar size being placed on top of each other (OT), is the absorption of nutrients by both partners of the graft. Necrotic tissue was only observed in the upper partner and it is possible that the upper tissue was failing to obtain sufficient nutrients to ensure normal cell functioning. Fujii et al., (1972)

noted that when two different pieces of callus were placed as described for the OT system, there was a reduction in proliferation of the upper tissue in some combinations, and they suggested that there was some breakdown in the supply of nutrients from the lower to the upper piece of callus. Ball (1970), however, using a system whereby autoradiographs determined the accumulation of a radioisotope by a non-radioactive tissue cultured on top of a radioactive tissue, postulated that cells of a recipient tissue (upper partner) can actively absorb substances from cells of a donor tissue (lower partner).

Although Fujii et al., (1972) did not find that grafting callus together resulted in growth inhibition of one partner by another, the results in this study would seem to imply that culturing callus derived from cv. Pentland Ivory with S. sparsipilum affects the growth rate of the former (Section 5.3). This could be due to competition for nutrients and water from the medium, the cells of cv. Pentland Ivory being competitively inferior to those of S. sparsipilum, or if the two species are capable of activating an incompatibility response if grafted together, growth inhibition could be associated with such a response.

The experimental work described in Section 5.4 was aimed at obtaining some indication of the extent of intermingling of the callus cells within a callus system comprised of cells derived from species of different ploidy. As reported in Section 5.4, it was noted that where callus was derived from the culture of tissue from a single species rather than two species, analysis of nuclear-DNA contents seemed to reveal the presence of only one population of

nuclear-DNA (Figs. 5.2A to 5.2D). These results were interpreted as an indication that the majority of cells at the time of sampling these callus cultures, were in the pre-DNA synthetic phase (G1 phase). Work on suspension cultures of Rosa species and Acer pseudoplatanus has shown that cells accumulate in G1 during the stationary phase of growth, whereas during exponential growth phase, G2 is the predominant phase of the cell cycle (King et al., 1977). Sree Ramulu et al., (1985) demonstrated the existence of a similar pattern in cell suspensions of the tetraploid potato cv. Bintje. As all callus systems in this experiment were sampled three to four days after subculturing, the inference from these results is that the majority of cells were still in a stationary phase of growth from the previous culture, and had not yet entered the lag phase where DNA replication would commence prior to exponential growth. Work on cell suspension cultures of Acer pseudoplatanus has shown that evidence of DNA replication is not apparent until day four of culture (Bayliss et al., 1974), and presumably this timing would vary with genotype and cultural conditions, so that in the case of the potato species examined in this experiment, it is possible that evidence of DNA replication would not appear until a later stage.

Fig. 5.2A (Section 5.4) is representative of the nuclear-DNA levels found in callus cultured for three months and derived from diploid stem internodal tissue grown in vitro (Section 4.2) and shows two major peaks occurring between ten and 30 units. Fig. 5.2B (Section 5.4) shows the nuclear-DNA levels found in callus of like origin but cultured for 13 months, with the majority of nuclei exhibiting DNA values between ten and 30 units. It would

appear from these results that there is no obvious change in ploidy level as a result of culture on media containing growth regulators. These results contrast with the findings of Sree Ramulu et al.,(1985) who found that both polyploidization and aneuploidy occurred during the early stages of callus induction in all genotypes of potato investigated. In addition, the authors found that further growth of callus led to increases in the frequency and extent of polyploidy.

Fig. 5.2C (Section 5.4) is representative of the nuclear-DNA content found in callus cultured for three months, and derived from tetraploid stem internodal tissue grown in vitro, and as with callus derived from the diploid species, there would appear to be two groupings of DNA levels, occurring between 40 and 60 units. These same groupings were evident when callus of the same origin, but cultured for 13 months, was analysed. Thus an increase in the length of culture (from three to thirteen months) has resulted in little change in the nuclear-DNA content. So it would appear as with callus of diploid origin, that extending the period of culture to 13 months has not resulted in any obvious change in ploidy level in the cells within the callus. There is some evidence that some callus systems do tend towards stability, often after an initial period of large variability in the DNA content of the nuclei, for example, Marchetti et al., (1976) using Nicotiana tabacum callus cultures could only identify tetraploid classes after two or three transfers.

The results obtained in analysing callus derived from tissue of single species origin suggest that there was no obvious

change in ploidy level with prolonged culture, and furthermore, that sampling of callus within a period of four days after sub-culture finds the majority of the cells in the callus in the G1 phase of the cell cycle. If such observations remain true within the mixed callus system then it might be possible to determine the extent to which callus derived from tissues of two species will mix when co-cultured.

Figs. 5.2E and 5.2F show the DNA distributions of mixed callus co-cultured (two callus explants, one on top of the other) for nine months. As noted in Section 5.4, a greater spread of DNA values was revealed by these mixed callus systems, and a comparison of these histograms with those representing diploid and tetraploid cells in culture could lead to the results being interpreted as portraying both diploid and tetraploid in the G1 phase. Further comparisons between the histograms representing these mixed callus systems (Figs. 5.2E and 5.2F) and those representing single callus cultures (Figs. 5.2A to 5.2D) reveal that a greater majority of nuclei are exhibiting DNA levels of a diploid nature rather than a tetraploid nature. This could imply that there is a tendency for the diploid cells to dominate a mixed callus system. There is evidence in the literature that diploid cells can have a selective advantage in culture over cells of other ploidy levels. In some plant species, for example, Prunus amygdalus, Daucus carota, Oryza sativa, Triticum aestivum, despite the presence of different ploidy levels in culture only or mostly diploid plants have been regenerated (D'Amato, 1978). Bayliss (1980) discussed the possibility that within a genetically heterogenous population of cells, the most

successful cell genotype will be those that reproduce most prolifically, suggesting differences in relative growth rate for cell numbers or mean generation time. For example, Blaschke et al., (1978) found that under the in vitro conditions established, the haploid cell cycle of cultured cells of Daucus carota appeared to be 20% shorter than the diploid one which was shorter than the tetraploid one. However, it has also been shown that with Daucus carota and Nicotiana tabacum, there were no differences in mean generation time between any of the lines examined, instead cell lines were influenced by the limiting growth factor in the medium. For example, low phosphate levels favoured the presence of the tetraploid cells whereas elimination of nutrient starvation, that is, frequent transfer to fresh medium favoured the diploid cells (Bayliss, 1980). If the suggestion made earlier that diploid cells are dominating these mixed callus systems is correct, then it is possible that conditions established in these mixed callus systems favoured the proliferation of the diploid component of the system.

Fig. 5.2G is representative of a mixed callus system where calluses of diploid and tetraploid origin have been cut into relatively small pieces and then randomly mixed together. A greater scattering of DNA values was present in this system, than in the systems represented by Figs. 5.2E and 5.2F. Figs. 5.2H, 5.2I and 5.2J are representative of the nuclear-DNA content of another mixed callus system, which involves the placing together of two callus explants both derived from species of different genotype, that is diploid and tetraploid. The selection of sampling points within this system was aimed at showing the extent to which the callus was

a composition of the two cell types, as the point at which the two callus explants were joined, was reached (Fig. 5.1, Section 5.4). Therefore if any dominance of one cell type was to be expected, it would be tetraploid cells in sample point one (Fig. 5.2H) with a combination of both tetraploid and diploid at sample point two (Fig. 5.21) and a possible dominance of diploid cells at sample point three (Fig. 5.2J). However, an examination of these three histograms shows a wide spread of DNA values occurring at all three sample points. This wide spread is suggestive of the presence of both diploid and tetraploid cells in both G1 and G2 phases of their cell cycles. Alternatively, the DNA distribution could be representative of cells mainly in the G1 phase, but cells that had undergone polyploidization and aneuploidy. As aneuploidy was detected in the roots derived from the regenerants of such a mixed callus system (Section 8.2), it is possible that some of the variation is due to real differences in chromosome numbers between individual cells.

There are slight differences between the three sample points as expressed by the histograms. Both Figs. 5.2H and 5.2I show nuclei with higher DNA content than that shown by the other histograms previously discussed, and the DNA content would appear to be equivalent to, and above that expressed by the tetraploid cells (Figs. 5.2C and 5.2D). On the other hand, Fig. 5.2J shows less of a scattering of DNA values, with some concentration of nuclei with DNA content towards the values held by the nuclei of the diploid cells, a trend which could be expected as this represents callus sampled from what originally was the diploid

component of the mixed callus system.

The results achieved through nuclear-DNA analysis do not allow any positive conclusion to be made with respect to the mixing of cell types within a mixed callus system. For such conclusions to be made accurate identification as to which phase of the cell cycle the cells are in prior to analysis would be necessary. There is also the possibility that endomitosis has taken place within the diploid tissue resulting in cells with a nuclear-DNA content equivalent to tetraploid cells, and thus presenting a misleading picture as to the cell populations present.

CHAPTER 6

GRAFTING OF DIFFERENTIATED TISSUE

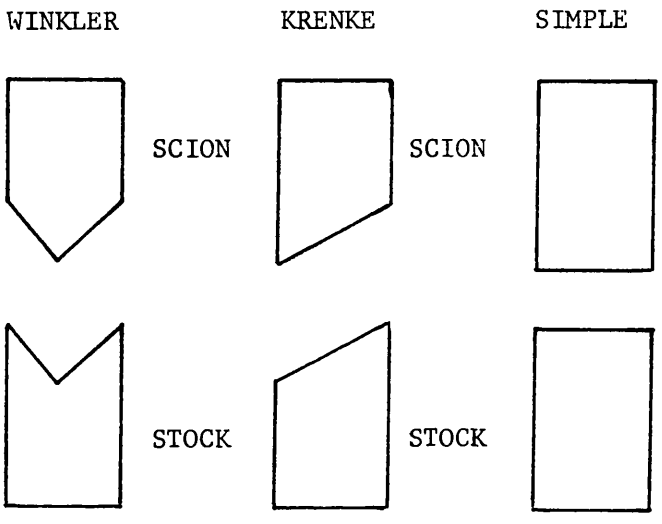
6.1. STEM INTERNODAL GRAFTING

6.1.1. Introduction

As detailed in the General Introduction (Chapter 1) grafting techniques have been used to achieve a combination of tissues from which chimeras have been regenerated (Winkler, 1970; Clayberg, 1974). It was decided to attempt similar grafting techniques with this aim in mind, and also to determine both the feasibility of performing grafts *in vitro*, and the level of compatibility between two *Solanum* species of different ploidy level.

All grafts were composed of stem internodal tissue, which was obtained from stems of actively growing plantlets derived from shoot-tip cultures. Different types of grafts were used in this study (Fig. 6.1). Firstly, Krenke's technique (1933) was employed whereby a diagonal cut is made across the width of the stem internode. A long slanting cut was then made at the basal end of the scion and a corresponding cut was made at the apical end of the stock. When the two sections were united, the cut surfaces were in contact.

Fig. 6.1.



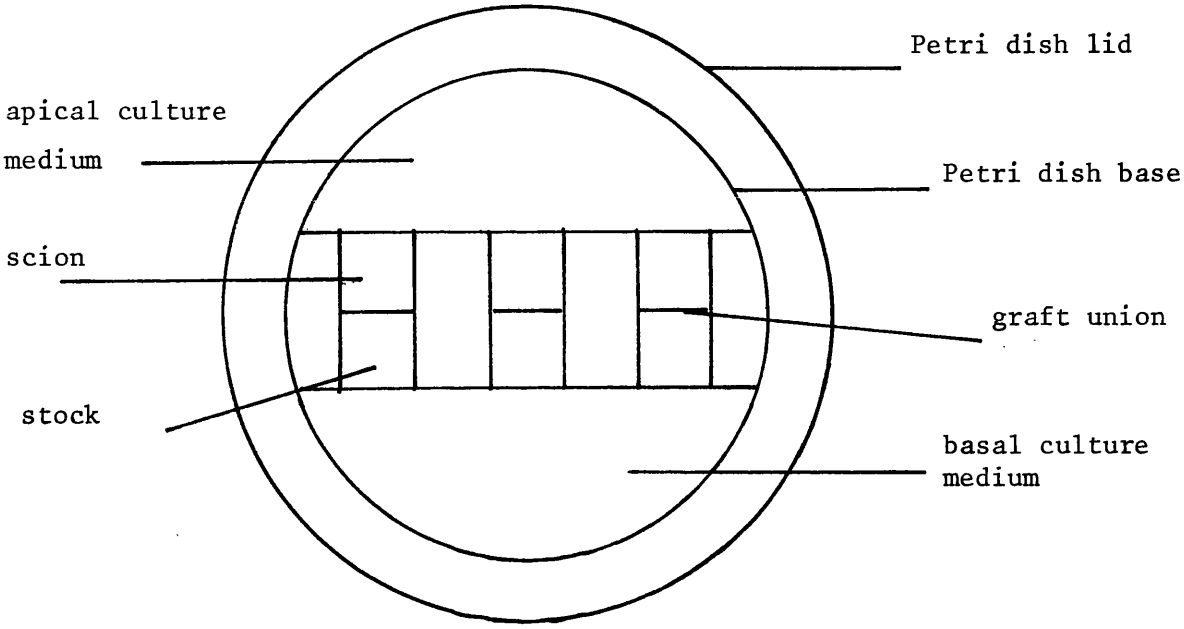
Secondly, a simple grafting technique was also used in which the internodal section was divided with a single cut at right angles to the axis. Uniting the two sections resulted in these cut surfaces achieving contact. Finally, the grafting technique of Winkler (1907) was used in this study, utilising a similar graft to that known horticulturally as a wedge or cleft graft. The internodal section was divided with a single cut at right angles to the axis. The scion was then prepared with its basal end in the form of a V-shape or wedge, and the stock was split at its apical end and sufficient tissue removed to accommodate the wedge shape of the scion.

The complete grafted explant varied between 8mm and 14mm in length, and between 2mm and 3mm in diameter. Where the length of the internode was insufficient to provide a grafted explant of this size, two different internodes from the same plantlet were used. To achieve good contact between the scion and stock, the scion must be of the same diameter as the stock.

During the course of one grafting experiment (Section 6.1.4) a "split-agar" petri dish was used, which was set up using the following procedure. The lower half of a 9cm sterile, plastic dish was divided into two halves with a piece of sterile aluminium box section (95 x 12mm) placed across the centre of the dish. An arrow was then marked on the outside of the base of the dish with a felt pen to act as a reference point to check the orientation of the dish. The appropriate molten agar medium ($15cm^3$) was poured into each half of the dish and allowed to solidify, after which the box section was carefully removed. The grafted internodal sections

were then inserted between two agar surfaces as shown in Fig. 6.2.

Fig. 6.2.



The petri dish was sealed with Parafilm and incubated either in a horizontal or vertical position, the latter to maintain the original orientation of the donor plant. Some internodal sections were reassembled inside a 12mm length of sterile silicone tubing (Portex, Hythe, Kent), as this ensured that the cut faces of the internodal sections were held together firmly. The procedure described was used by Parkinson et al., (1982) for the formation of successful autografts of Lycopersicon esculentum, Datura stramonium and Nicandra physaloides. Assessment of graft development was on a mechanical basis, made by attempting to pull apart the stock and scion using fine forceps.

6.1.2. Effect of grafting technique on graft development and formation

The aim of this investigation was to assess the possibility of grafting stem internode sections *in vitro*, using a procedure based on the *in vivo* techniques established by Winkler (1907) and Krenke (1933).

Grafted internodes were inoculated onto basal semi-solid medium (Section 2.2) to which 3% sucrose (w/v), kinetin ($0.2mgl^{-1}$) and indole-3-acetic acid ($0.2mgl^{-1}$ and $2.0mgl^{-1}$) were added. These combinations of growth regulators were used by Parkinson *et al.*, (1982) to establish grafts between internodal sections *in vitro* (Section 1.3.2). *S. tuberosum* ssp *tuberosum* cv. Pentland Ivory and cv. Fortyfold, and *S. sparsipilum* were used, and 108 autografts of each were constructed, 54 of the Winkler type and 54 of the Krenke type. Culture procedure and conditions were as described in Section 6.1.1. All cultures were incubated under 16h daylength at a light intensity of $85\mu Mm^{-2}s^{-1}$ PAR and a temperature of $22 \pm 1^{o}C$ (HL/LT).

Cultures were assessed after one, two and four weeks. After seven days, prominant callus proliferation and swelling at the base of each internode had occurred. Between seven and ten days of culture, roots and shoots had developed from both the stock and scion, and as roots developed there was a tendency for the scion and stock to be forced apart, an effect that was more pronounced in grafts ofthe Krenke type compared to those of the Winkler type. The data from this investigation are given in Table 6.1. Of the 324 grafts assessed, 9% had achieved a degree of mechanical strength at the graft union, that is, they could not be pulled apart.

Of this 9%, 59% were Winkler grafts and 41% were Krenke grafts. After four weeks of culture, there was no further increase in the number of grafts with strong unions.

6.1.2.1. Effect of IAA concentration in the culture medium on graft development and formation

In addition to evaluating the effectiveness of the two different grafting techniques, the experiment as described in Section 6.1.2, gave an indication of the explant response, both in terms of graft formation and morphogenetic behaviour after four weeks of culture on media containing different concentrations of IAA. It would appear from the results (Table 6.1) that the formation of successful autografts is linked to auxin concentration in the medium; the optimum result with respect to graft establishment was achieved on medium containing 2.0mgl^{-1} IAA (Table 6.1).

TABLE 6.1. Effect of grafting technique and IAA concentration in the culture medium on graft development in autografts of potato species

SPECIES AND CULTIVAR	IAA CONCN. (mgl^{-1})	WINKLER GRAFTS					KRENKE GRAFTS				
		% SCIONS WITH ROOTS	% SCIONS WITH SHOOTS	% STOCKS WITH ROOTS	% STOCKS WITH SHOOTS	% GRAFTS ESTAB-LISHED	% SCIONS WITH ROOTS	% SCIONS WITH SHOOTS	% STOCKS WITH ROOTS	% STOCKS WITH SHOOTS	% GRAFTS ESTAB-LISHED
cv. Forty-fold	0	-	-	17	17	-	50	-	55	17	17
	0.2	39	-	28	-	-	83	17	50	17	-
	2.0	-	-	33	-	-	67	-	33	-	33
cv. Pent-land Ivory	0	-	11	-	17	-	-	-	11	22	-
	0.2	89	-	78	22	33	67	33	83	67	-
	2.0	67	-	78	-	28	83	-	89	33	-
S.spars-ipilum	0	17	-	22	17	17	-	-	11	5	-
	0.2	-	-	44	33	-	17	-	33	17	-
	2.0	17	17	39	-	17	17	17	33	17	17

Key:

Media: MS + 3% sucrose (w/v) + 0.2mgl^{-1} kinetin + 1AA

(0, 0.2 or 2.0mgl^{-1})

Temperature: 22 ± 1^{o}C

Light conditions: 16h daylength; 85μMm^{-2}s^{-1} PAR

Number of replicates per treatment: 18

Period of culture: 4 weeks

6.1.3. Use of split-agar dish

This experiment was designed to establish the influence that differing concentrations of auxin applied to the apical and basal ends of the internode explants would have on graft formation. Parkinson et al. (1982) found that an absolute requirement for the formation of a successful graft is the application of IAA to the apical end of the internode.

Autografts of the Winkler type were constructed using two Solanum species: S. tuberosum ssp tuberosum cv. Pentland Ivory and S. sparsipilum. Using the culture technique described for split-agar dish in Section 6.1.1, the concentrations of IAA (0, 0.2 or 2.0mgl^{-1}) in the apical and basal sections of the Petri dish were either equal or different (Table 6.2). Other culture conditions were as detailed in Section 6.1.2. Each medium treatment consisted of 16 autografts per species, eight incubated in a horizontal position and eight in a vertical position,. Results were assessed after one, two and four weeks of culture and they are listed in Table 6.2.

Swelling of the internodes, and callus formation at the graft union and base of the internodes occurred between day three and day seven. Of the 288 grafts, 26% had achieved sufficient contact to resist pulling apart, indicating that the use of split-agar Petri dishes had markedly increased the number of explants with strong graft unions: of this 26%, 52% were incubated horizontally and 48% vertically. When the explants were incubated vertically, absence of auxin in the apical medium resulted in no graft establishment; with horizontal incubation however, zero graft establishment

occurred only when both apical and basal media lacked auxin. With both species, the presence of $0.2mgl^{-7}$ IAA in the apical medium favoured graft establishment; a slight decrease in the number of successful grafts occurred when the apical medium contained $2.0mgl^{-1}$ IAA.

Key:

Media: MS + 3% sucrose + kinetin ($0.2mgl^{-1}$) + varying concentrations of IAA (as shown)

Temperature: $22 \pm 1^{o}C$

Light conditions: 16h daylength; $85\mu Mm^{-2}s^{-1}$PAR

Number of replicates per treatment: 16

Period of culture: 4 weeks

TABLE 6.2. Effect of differing apical and basal concentrations of auxin (IAA) on graft development in stem autografts of potato

SPECIES AND CULTIVAR	IAA CONCN. APICAL/ BASAL MGL^{-1}	HORIZONTAL INCUBATION			VERTICAL INCUBATION		
		% EXPLANTS WITH CALLUS AT GRAFT UNION	% EXPLANTS WITH CALLUS AT BASE OF INTERNODE	% GRAFTS ESTABLISHED	% EXPLANTS WITH CALLUS AT GRAFT UNION	% EXPLANTS WITH CALLUS AT BASE OF INTERNODE	% GRAFTS ESTABLISHED
S.tuberosum ssp. *tuberosum* cv. Pentland Ivory	0/0	100	100	0	37	50	0
	0/0.2	37	37	25	25	37	0
	0/2.0	50	62	25	37	37	0
	0.2/0	12	12	25	50	62	37
	0.2/0.2	37	50	37	37	50	50
	0.2/2.0	87	100	50	37	75	25
	2.0/0	87	87	25	37	62	37
	2.0/0.2	62	75	25	25	37	50
	2.0/2.0	75	75	25	12	25	37
S.sparsipilum	0/0	87	100	0	50	50	0
	0/0.2	12	25	37	0	12	0
	0/2.0	37	37	25	25	37	0
	0.2/0	0	0	37	87	100	50
	0.2/0.2	75	75	25	50	62	50
	0.2/2.0	75	75	37	75	87	25
	2.0/0	87	100	12	50	50	37
	2.0/0.2	12	12	25	25	25	37
	2.0/2.0	25	25	50	50	62	12

6.1.4. Importance of close tissue contact to graft development

The aim of this investigation was to evaluate the importance for graft establishment, of the cut surfaces of the scion and stock being held together firmly.

The culture technique and conditions were as detailed in Section 6.1.3. Simple grafts and Winkler grafts were carried out on internodal sections which were then reassembled inside sterile, silicone tubing (Section 6.1.1). Each medium treatment per species consisted of 24 autografts: 12 incubated horizontally and 12 incubated vertically. For each species, six autografts were of the Simple type and six of the Winkler type.

As described in Section 6.1.3, swelling of the internodes, and callus formation at the graft union and base of the internodes had occurred by day seven. Of the grafts cultured, 42% resisted pulling apart, and of this 42%, 62% were of the Winkler type and 38% of the Simple type. The data for this experiment are listed in Table 6.3 and as the results show, grafting success was not markedly favoured by either horizontal or vertical incubation. Furthermore, differing concentrations of IAA in the apical and basal sections of the petri dishes appeared to have no effect on grafting success, except that an absence of auxin in the apical medium appears to inhibit graft formation.

The autografts in this experiment were subjected to the same conditions as the autografts in Section 6.1.3, except that contact between the graft partners was not ensured with the latter as

it was with the former. As greater grafting success, in terms of percentage grafts established, was achieved when tissue contact was ensured, it would appear that close tissue contact is an important requisite of graft formation.

Key:

Media: MS + 3% sucrose + kinetin ($0.2mgl^{-1}$) + varying concentrations of IAA (as shown)

Temperature: $22 \pm 1^{o}C$

Light conditions: 16h daylength; $85\mu Mm^{-2}s^{-1}$ PAR

Number of replicates per treatment: 24

Period of culture: 4 weeks

TABLE 6.3. Influence of close tissue contact on graft development in stem autografts of potato

SPECIES AND CULTIVAR	IAA CONCENTRATION APICAL/BASAL (MGL^{-1})	HORIZONTAL INCUBATION		VERTICAL INCUBATION	
		% GRAFTS ESTABLISHED - WINKLER	% GRAFTS ESTABLISHED - SIMPLE	% GRAFTS ESTABLISHED - WINKLER	% GRAFTS ESTABLISHED - SIMPLE
S. tuberosum ssp. tuberosum cv. Pentland Ivory	0/0	-	-	-	-
	0/0.2	-	-	-	-
	0/2.0	33	-	-	-
	0.2/0	100	83	100	50
	0.2/0.2	83	67	83	33
	0.2/2.0	50	33	67	17
	2.0/0	100	50	67	83
	2.0/0.2	67	50	67	67
	2.0/2.0	67	33	67	50
S. sparsipilum	0/0	-	-	-	-
	0/0.2	-	-	-	-
	0/2.0	17	-	-	-
	0.2/0	83	33	100	33
	0.2/0.2	67	33	83	33
	0.2/2.0	50	33	50	33
	2.0/0	100	67	100	83
	2.0/0.2	67	33	83	67
	2.0/2.0	50	33	67	33

6.1.5. DISCUSSION

The results of the experiments described in Chapter 6 have focussed attention on two factors seen by workers as important in determining the success or failure of a graft. Firstly, there is the importance of tissue contact which Garner (1979) sees as one of two criteria which must be satisfied prior to successful graft formation. The second factor deemed to be important in successful graft formation is the availability of auxin.

With respect to tissue contact several workers have stressed the importance of adequate contact between stock and scion in the process of graft formation: for example, Roberts *et al.*, 1961; Colin *et al.*, 1976; Yeoman *et al.*, 1978; Moore, 1984. The existence of a minimum area of tissue contact was suggested by results obtained by Huang *et al.*, (1980) during work on *in vitro* micrografting of apple shoot-tips (*Malus domestica* Borkh). Huang *et al.*, (1980) found that the probability of successful graft unions increased with the size of the scion piece; the percentage of successful graft unions was 15% when the scion consisted of an apical dome only and increased to 65% when the scion consisted of an apical dome and two leaf primordia. It is likely that this minimum area of tissue requirement reflects not only the need for a minimum degree of tissue contact, but also importance of an auxin supply, which in the case of these micrografts would be provided by the leaf primordia.

The results achieved during the experiment described in Section 6.1.4 have confirmed the need for close tissue contact in graft development. Reassembling the potato autografts in sterile,

silicone tubing ensured tissue contact was maintained, and this process resulted in 42% graft establishment, whereas prior to using this technique, 26% was the maximum graft formation achieved. In addition throughout all the experiments in Chapter 6, it was the Winkler graft, rather than the Krenke or Simple graft, which resulted in the greater number of successful graft unions. Of these three grafts, contact between as large an area of tissue as possible is ensured by the wedge shape of the Winkler graft. Furthermore, the interlocking parts of the stock and scion in a Winkler graft would prevent independent movement better than the mere transverse cuts seen in the Krenke and Simple grafts.

The presence of auxin is considered by several investigators as important for the promotion of graft formation (Shimomura et al., 1978; Parkinson et al., 1982), and its function, it would seem is to encourage vascular differentiation. As described in Section 1.3.1, differentiation of vascular tissue across the graft interface is the final phase in the process of graft development and as a result, the mechanical strength of the graft is increased, mainly owing to the presence of xylem elements whose formation is strongly limited by auxin level (Jacobs, 1956) The role of auxin in inducing xylem differentiation has been considered by many workers. For example, Jacobs (1952), in work with Coleus blumei Benth found that removal of all auxin sources, that is, leaves and apical buds, reduced the amount of xylem regeneration forming around a wound to a highly significant degree. Similarly, Aloni et al., (1985) found that vascular regeneration around wounds in seedlings of Zea mays L cv. Jubilee F_1 was related to the level of auxin present in the area of the wound. With respect to grafting,

the formation of graft unions in Lycopersicon esculentum was facilitated through leaving a bud or leaf on the scion (Lyndsay, 1972).

Results as reported in Sections 6.1.2.1 and 6.1.3. indicate that the presence of auxin is a critical factor in the process of graft formation. In both experiments, the lowest numbers of grafts were formed when the medium was lacking in auxin and in one experiment (Section 6.1.2.1) the maximum number of grafts were found when the explants had been cultured on media containing the highest concentration of auxin ($2.0 mgl^{-1}$). The results obtained using a split-agar dish (Fig. 6.2, Section 6.1.1), a method employed by Parkinson et al., (1982) to facilitate apical application of auxin, are reported in Section 6.1.3. Parkinson et al., (1982) found that while essential nutrients could be fed either basally or apically, it was vital that auxin was applied apically to the scion if graft formation in excised internodes of Lycopersicon esculentum Nicandra physaloides and Datura stramonium, cultured in vitro, was to be achieved. Although auxin can be transported acropetally, the movement of auxin in the plant tissue generally is in a basipetal direction (Jacobs, 1977). Consequently Parkinson et al., (1982) see the lack of auxin in grafted internodes, because of the low acropetal auxin movement, as contributing to the small number of vascular strands forming across the graft union in individuals in which auxin is not applied apically.

Sections were not taken of the internodal grafts established during these experiments (Sections 6.1.3 and 6.1.4).

Therefore, any statements as to vascular differentiation cannot be confirmed by microscopic evidence. However, as Table 6.2 and 6.3 show, the presence of auxin in the apical medium is essential for the establishment of strong graft unions when excised internodes are incubated vertically, in that lack of auxin (IAA) in the apical medium resulted in zero graft establishment. There were some successful autografts achieved however, when auxin was present in the basal medium but absent from the apical medium, and when the excised internodes were incubated horizontally (Table 6.2). As has already been discussed, acropetal movement of auxin does occur and furthermore, gravity will affect basipetal movement, in that when a normally erect organ is placed horizontally, or inverted, then the velocity of the basipetal auxin transport is reduced (Wareing et al., 1981). Assuming horizontal incubation of the explant and basal application of auxin promotes acropetal movement of auxin, it is debatable as to the extent to which this acropetal movement can induce vascular regeneration. Parkinson et al., (1982) reported on the presence of vascular connections when the apical medium contained zero auxin, but, they did not state whether these limited vascular connections would be sufficient to produce a graft of adequate tensile strength.

A further consideration must be the possibility that the success of the grafts in this experiment was the result of cohesion, which occurs in the initial stages of grafting, independent from vascular differentiation, which occurs in the later stages, and as far as is known, takes place regardless of auxin level. It is possible to achieve grafts whose strength at the graft union is not

due to the presence of vascular tissue across the graft union. For example, it was found that *in vitro* grafts between *Coleus* internodes were lacking in cellular division and vascular differentiation yet partners of the graft adhered tenaciously (Stoddard *et al.*, 1980). In addition, there have been reports of scions functioning in the absence of vascular continuity (Herrero, 1951; Musik, 1958).

Parkinson *et al.*, (1982) found that the formation of vascular connections was favoured by levels of 0.2 and 2.0mgl^{-1} IAA in the apical medium and zero levels of auxin in the basal medium; any auxin present in the basal medium reduced the number of vascular connections induced by the apical application of the auxin. This requirement for an auxin gradient was evident in a large proportion of the autografts investigated in Section 6.1.4, the general pattern being that as auxin concentration in the basal medium increased, percentage graft establishment decreased. There were inconsistencies to this general rule, for example, with autografts of cv. Pentland Ivory incubated vertically, the percentage graft established was 67 when the apical medium contained 2mgl^{-1} IAA regardless of the auxin content in the basal medium (Table 6.3). Such inconsistencies can probably be explained in terms of endogenous levels of auxin and possibly other hormones, together with the physiological state of the experimental system. Referring to Section 6.1.3 and Table 6.2, the response (as seen in Section 6.1.4) to an auxin gradient is far less apparent. Again this could be the result of other factors, such as endogenous hormone levels and/or the physiological state of the explants, however, it is possible that in Section 6.1.4, where the grafts were contained within the sterile, silicone tubing,

the criteria of tissue contact was satisfied, thus allowing the grafts, as biological systems, to be more responsive to other conditions, such as exogenous auxin levels.

The experiments described in this chapter and the results obtained, have shown that graft formation is facilitated by both the apical application of auxin and the maintenance of close tissue contact between the partners of the graft.

6.2. THIN CELL LAYER GRAFTING

6.2.1. Introduction

This investigation was concerned with the feasibility of excising a thin layer of some three to six cells thick from stem tissue (epidermal layer), and then providing the conditions necessary for that thin layer to establish a graft union with stem tissue of differing ploidy level. If the thin cell layer was not rejected by the stem tissue and a form of graft union occurred between the two tissues, the next step in the process of potential chimera production could be to determine the possibility of encouraging regeneration from this grafted system. It was envisaged that the grafting on of a thin cell layer might not be too dissimilar to grafting on the L1 layer, and thus regeneration from the grafted area could favour the development of a periclinal chimera. As the terms stock and scion cannot be strictly applied to the tissues used in these experiments, that is, the thin cell layer and stem tissues, the terms donor tissue and recipient tissue have been substituted.

In deciding on the growth regulator content of the culture medium, consideration had to be given to providing conditions for encouraging graft formation, yet at the same time, such conditions must not result in inhibition of regeneration at a later stage. Work by Tran Thanh Van et al., (1974) has shown that epidermal cells have the potential to be flexible in their morphogenetic expression, and that this expression is determined, to a large extent, by the auxin and cytokinin levels present in the culture medium.

With these factors in mind, it was decided to use combinations of growth regulators that had been shown to favour regeneration in potato, for example, NAA and BAP, (Roca et al., 1974; Webb et al., 1972); NAA and kinetin, (Wang et al., 1975; Novak et al., 1980). IAA was omitted because of the ease with which it is broken down by plant tissue, a problem that might be exacerbated because of increased peroxidase activity resulting from the wounding of the plant tissue (Andreae et al., 1960). 2,4-D was substituted for NAA in two experiments (Section 6.2.8 and 6.2.9) to evaluate whether its high level of activity, and its ability to induce rapid callus proliferation would have a favourable effect on graft formation.

6.2.2. Experimental procedure

Epidermal strips, composed of three to six cell layers were excised from the stems of plantlets derived from shoot-tip cultures. In the case of autografts, these thin cell layers were replaced onto the section of the stem from which they had been removed, either maintaining or reversing the original polarity. The possibility of successful heterografts was also investigated, and here thin cell layers were placed onto stem tissue of differing ploidy level, from which a similar layer had been removed, in such a way as to maintain the original polarity. With some explants, the donor tissue (thin cell layer) covered the entire length of the recipient tissue and the final length of the explant was 6mm to 10mm. With other explants, the donor tissue occupied approximately 60% of the length of the recipient tissue and the final length of the explant was 15mm to 20mm.

Graft success was assessed in a number of ways. Using a fine needle and fine forceps, attempts were made mechanically to remove the donor tissue, and those that resisted removal were sectioned to examine continuity between donor tissue and recipient tissue. Where sectioning was used merely as a tool in graft assessment, a simple procedure was followed: sections taken from pith-mounted explants were immersed in Paragon stain for 2 to 3 min. and then examined under a microscope. Where more accurate histological analysis was intended, sectioning was carried out as described in Section 2.6.

6.2.3. Influence of explant position on agar on graft development

This was a preliminary investigation into the practicability and effectiveness of this grafting procedure, and the effect of the position of the explant on agar on graft development.

Basic culture techniques were as described in Section 6.2.2. All grafts were autografts and the species and cultivars used in this investigation were S. tuberosum ssp tuberosum cv. Desiree and cv. Fortyfold. The basal culture medium (Section 2.2) was supplemented with 3% sucrose (w/v) and NAA and BAP in varying concentrations ranging from 0.5 to 2.0mgl^{-1}. Cultures were incubated under a 16h daylength at a light intensity of 85μMm^{-2}s^{-1}PAR and a temperature of 22 ± 1^{o}C. The donor tissue covered the entire length (6mm to 10mm) of the recipient tissue, and the subsequent autografts were cultured in four different positions:

1. Donor tissue excised and replaced with polarity maintained; explant incubated horizontally on medium with grafted area

uppermost.

2. As in (1) but with polarity reversed.
3. As in (1) but with the grafted area in contact with the medium.
4. As in (1) but with the explant incubated in a vertical position, that is, inserted into the agar so that the original morphological polarity of the tissue was maintained.

For each combination of NAA and BAP, 24 autografts were cultured in these different positions, so that for each cultivar 384 autografts were investigated. Results were assessed after one week and four weeks (Table 6.4).

After seven days all explants had expanded, however, the extent of expansion varied with concentration of growth regulators in the medium. At this stage, no callus had developed and few explants had shown an organogenic response. Some explants had discoloured, and this discolouration occurred where explants had been cultured on media with a higher cytokinin than auxin level. After four weeks of culture, all explants, except those cultured on media lacking in growth regulators, had developed callus. The majority of explants had developed roots and a few had produced shoots. Of the grafts cultured, 28% were successful. There was no combination of growth regulators that was clearly promoting graft formation, however, grafts failed to develop when either auxin or cytokinin was absent. Furthermore, graft development was seemingly favoured by comparatively high levels of growth regulators (see Table 6.4).

No grafts were successful when the donor tissue was replaced onto the recipient tissue with its polarity reversed. Position of the grafted explant on the agar only slightly affected grafting success with 25% of grafts successful when cultured as described for position one, and 21% when cultured as described for position four. Of the grafts cultured as described for position three, only 10% were successful. Position of the grafted explant on the agar seemed to affect regeneration, in that, there was less root and shoot production from the recipient tissue when the grafted explant was inoculated onto agar in a horizontal position (position one).

TABLE 6.4. INFLUENCE OF EXPLANT POSITION ON AGAR ON GRAFT DEVELOPMENT IN THIN CELL LAYER AUTOGRAFTS OF POTATO

CULTIVAR	GROWTH REGULATOR CONCN. IN MEDIUM (MGL^{-1})		% GRAFT ESTABLISHMENT			
	NAA	BAP	POSITION (1)	POSITION (2)	POSITION (3)	POSITION (4)
Desiree	0	0	-	-	-	-
	0	0.5	-	-	-	-
	0	1.0	-	-	-	-
	0	2.0	-	-	-	-
Fortyfold	0	0	-	-	-	-
	0	0.5	-	-	-	-
	0	1.0	-	-	-	-
	0	2.0	-	-	-	-
Desiree	0.5	0	-	-	-	-
	0.5	0.5	-	-	17	-
	0.5	1.0	17	-	17	17
	0.5	2.0	50	-	17	33
Fortyfold	0.5	0	-	-	-	-
	0.5	0.5	33	-	-	17
	0.5	1.0	33	-	17	17
	0.5	2.0	83	-	33	67
Desiree	1.0	0	-	-	-	-
	1.0	0.5	33	-	-	17
	1.0	1.0	67	-	-	17
	1.0	2.0	67	-	33	50

TABLE 6.4 (CONTINUED). INFLUENCE OF EXPLANT POSITION ON AGAR ON GRAFT DEVELOPMENT IN THIN CELL LAYER AUTOGRAFTS OF POTATO

CULTIVAR	GROWTH REGULATOR CONCN. IN MEDIUM (MGL^{-1})		% GRAFT ESTABLISHMENT			
	NAA	BAP	POSITION (1)	POSITION (2)	POSITION (3)	POSITION (4)
Fortyfold	1.0	0	-	-	-	-
	1.0	0.5	17	-	17	33
	1.0	1.0	33	-	17	50
	1.0	2.0	83	-	33	83
Desiree	2.0	0	-	-	-	-
	2.0	0.5	33	-	-	17
	2.0	1.0	17	-	17	17
	2.0	2.0	83	-	33	67
Fortyfold	2.0	0	-	-	-	-
	2.0	0.5	33	-	17	33
	2.0	1.0	33	-	17	100
	2.0	2.0	83	-	33	33

Key:

Media: MS + 3% sucrose (w/v) with combinations of NAA and BAP as detailed in Table 6.4.

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Number of replicates per position: 6

Period of culture: 4 weeks

Position (1): Donor tissue excised and replaced with polarity maintained; explant incubated horizontally on medium with grafted area uppermost

Position (2): As in (1) but with polarity reversed

Position (3): As in (1) but with grafted area in contact with the medium

Position (4): As in (1) but with the explant incubated in a vertical position, that is, inserted into the agar so that the original morphological polarity of the tissue was maintained

6.2.4. Size of graft in relation to total size of explant

Culture technique and conditions were as described in Section 6.2.3 except that the grafted donor tissue occupied approximately 60% of the recipient tissue and the final length of the grafted explant was 15mm to 20mm. In addition, all explants were inoculated onto agar as described in position one (Section 6.2.3). Results were assessed after 14 and 28 days of culture.

The morphogenetic response of the explants followed a similar pattern to that outlined in Section 6.2.3. Callus production was visible in most explants after 14 days, mainly around the grafted area and at the ends of the recipient tissue. As Table 6.5 shows the number of grafts achieving a successful union in this experiment was low (7%). Organogenesis was the response most commonly observed in this experiment. In comparing these results with those obtained in Section 6.2.3, it would appear that graft establishment is favoured when the size of the grafted area is equal to the total length of the grafted explant.

TABLE 6.5. Effect of differing concentrations of NAA and BAP on organogenesis and graft development when size of grafted area was less than total length of explant

CULTIVAR USED	GROWTH REGULATOR CONCENTRATION IN MEDIUM (MGL^{-1})		% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS	% GRAFT ESTABLISH-MENT
	NAA	BAP			
cv. Desiree	-	-	33	25	-
	-	0.5	17	25	-
	-	1.0	25	-	-
	-	2.0	-	17	-
cv. Fortyfold	-	-	8	8	-
	-	0.5	17	-	-
	-	1.0	17	17	-
	-	2.0	25	25	-
cv. Desiree	0.5	-	17	42	-
	0.5	0.5	17	42	17
	0 5	1.0	-	50	-
	0.5	2.0	17	42	-
cv. Fortyfold	0.5	-	-	-	-
	0.5	0.5	8	17	-
	0.5	1.0	17	17	-
	0.5	2.0	25	25	-
cv. Desiree	1.0	-	25	50	-
	1.0	0.5	-	33	17
	1.0	1.0	8	42	25
	1.0	2.0	25	50	-

TABLE 6.5. (continued)

CULTIVAR USED	GROWTH REGULATOR CONCENTRATION IN MEDIUM (MGL^{-1})		% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS	% GRAFT ESTABLISH-MENT
	NAA	BAP			
cv. Fortyfold	1.0	-	8	42	-
	1.0	0.5	17	33	25
	1.0	1.0	17	17	-
	1.0	2.0	25	17	-
cv. Desiree	2.0	-	-	50	-
	2.0	0.5	-	33	25
	2.0	1.0	-	50	-
	2.0	2.0	8	8	17
cv. Fortyfold	2.0	-	-	50	-
	2.0	0.5	-	50	-
	2.0	1.0	-	17	-
	2.0	2.0	-	8	17

Key:

Media: MS + 3% sucrose (w/v) with combinations of NAA and BAP as detailed in Table 6.2.

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Number of replicates per treatment: 12

Type of graft: autograft

Period of culture: four weeks

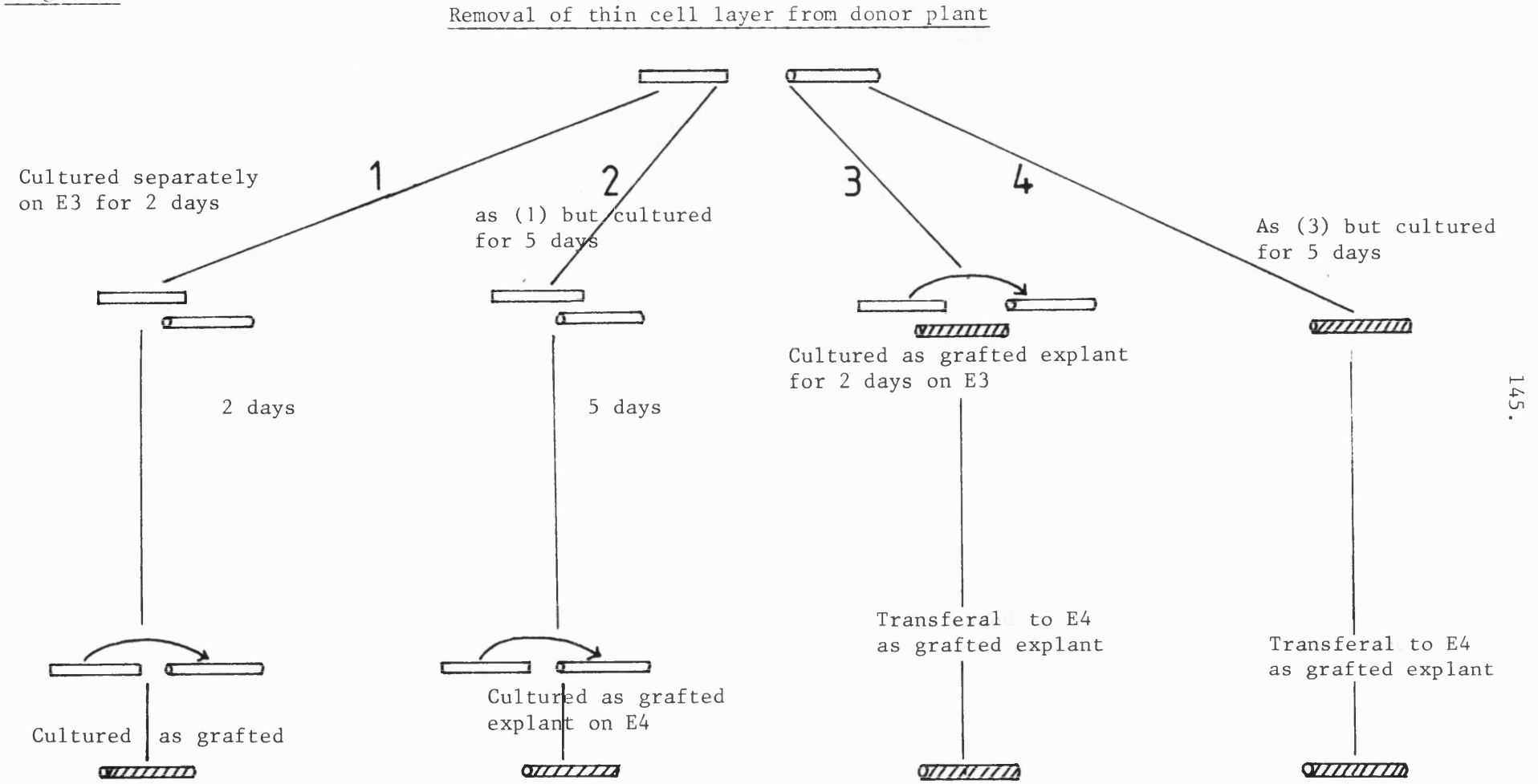
6.2.5. The use of a two-stage procedure

The main aim of this experiment was to alleviate the trauma caused by removal of a thin cell layer (donor tissue) from the parent plant, and in doing so, encourage subsequent graft formation. This was attempted in three ways involving the use of a two-stage procedure (E3/E4), low light and removal of the donor tissue followed by a period of culture prior to replacement onto the recipient tissue (see Fig. 6.3).

Basic culture techniques and conditions were as described in Sections 6.2.2 and 6.2.3. All grafts were autografts and were cultured horizontally with the grafted area uppermost, on solid agar under 16h daylength at two differing light intensities (see Fig. 6.3 for details of media and light conditions). The species and cultivars used in this experiment were S. brevidens, S. tuberosum ssp tuberosum cv. Fortyfold and cv. Pentland Ivory. There were ten autografts prepared for every variable that was investigated. Results were assessed after 15 and 30 days of culture.

All explants developed callus, either at one end of the recipient tissue, all over the explant, or at both ends of the recipient tissue. Incubation in low light appeared to increase the extent of callus production. With some autografts the donor tissue had seemingly established a firm union with the recipient tissue through callus production, however, this union was not sufficiently strong to resist removal of the donor tissue with forceps. It was observed that the donor tissue of some explants was capable of expansion whilst on top of the recipient tissue, without the establish-

Fig. 6.3.


Removal of thin cell layer from donor plant
1
2
3
4
Cultured separately on E3 for 2 days
as (1) but cultured for 5 days
As (3) but cultured for 5 days
Cultured as grafted explant for 2 days on E3
2 days
5 days
Transferal to E4 as grafted explant
Transferal to E4 as grafted explant
Cultured as grafted explant on E4
Cultured as grafted explant on E4

Key:

 : thin cell layer; donor tissue

 : stem tissue from which thin cell layer has been removed

 : grafted explant

Media: MS + 3% sucrose (w/v) + $1gl^{-1}$ casein hydrolysate + $2mgl^{-1}$ zeatin + $0.02mgl^{-1}$ 2,4-D; 2,4-D is not present in E4

Temperature: 22 ± 1°C

Light conditions: 16h daylength; $85\mu Mm^{-2}s^{-1}$ PAR (HL); $20\mu Mm^{-2}s^{-1}$ PAR (LL)

Number of replicates per treatment: 10

Period of culture: 30 days

Species and cultivars used: S. tuberosum ssp tuberosum cv. Fortyfold and cv. Pentland Ivory; S. brevidens

ment of any visible contact. Regeneration of roots and shoots occurred to a lesser degree than in other experiments, and originated from the ends of the recipient tissue. No autografts in this investigation were successful.

6.2.6. The use of IAA as a dipping solution

In this investigation autografts were cultured on media lacking in growth regulators, however, prior to culture, all or part of the autograft was dipped in a solution containing IAA. Care was taken during the course of this experiment that all procedures, prior to incubation of the explants under artificial light in the growth cabinet, took place in the dark; the aim being to minimise any reduction in IAA activity by the action of light until both donor and recipient tissues were in a "graft" situation. This experiment was set up in an attempt to try and encourage greater cell activity from the donor tissue itself. Furthermore, absence of growth regulators in the culture medium should discourage organogenesis and extensive callus development, and therefore, possibly facilitate the healing process which may be necessary for the formation of a firm graft union.

Basic experimental procedure was as outlined in Section 6.2.2. Two solutions of IAA were used, one containing $0.2mgl^{-1}$, the other containing $2.0mgl^{-1}$, and either the donor tissue or the donor tissue and recipient tissue (stem tissue less thin cell layer) were dipped in these solutions for a period of 60 seconds, prior to culture as autografts on basal semi-solid medium (Section 2.2) supplemented

with 3% sucrose (w/v). The species and cultivars used in this investigation were S. tuberosum ssp tuberosum cv. Fortyfold and cv. Pentland Ivory and S. sparsipilum; for each species and cultivar and IAA concentration, 40 autografts were assembled, 20 for each light intensity (20 and 85$\mu Mm^{-2}s^{-1}$PAR). Of the 20 explants, ten had only the donor tissue dipped in IAA solution and ten had both the donor tissue and recipient tissue dipped in IAA solution.

Grafts were assessed after 28 days of culture. The majority of explants had produced callus, which had developed from the sides of the recipient tissue. Development of donor tissue took the form of expansion, 31% under 85$\mu Mm^{-2}s^{-1}$PAR and 16% under 20$\mu Mm^{-2}s^{-1}$PAR. It was apparent that the initial stages of expansion had resulted in dislodging the donor tissue from its position on top of the recipient tissue with some explants (27%). These donor tissue explants did not continue their development once contact with recipient tissue had ceased to exist. Graft assessment revealed that no strong unions were formed by any of the autografts.

6.2.7. Graft response on media containing NAA in combination with kinetin

The aim of this investigation was to assess the influence this combination of growth regulators had in encouraging graft formation between diploid and tetraploid tissue, that is hetero-grafts.

Culture technique and conditions were as described in

Sections 6.2.2 and 6.2.3. The culture medium used was as outlined in Section 2.2, but supplemented with 3% sucrose (w/v) and varying concentrations of NAA ($0.2mgl^{-1}$ and $2.0mgl^{-1}$) in combination with kinetin ($0.2mgl^{-1}$ and $2.0mgl^{-1}$).

The species and cultivars used in this investigation were S. tuberosum ssp tuberosum cv. Pentland Ivory and cv. Majestic and S. sparsipilum. Explants were inoculated horizontally onto agar, with the grafted surface uppermost and with the donor tissue covering the entire length of the recipient tissue. Two different light and temperature regimes were used: low light/high temperature (LL/HT) and high light/low temperature (HL/LT). For each medium combination and light/temperature regime, there were 12 heterografts. Results were assessed after 14 and 28 days of culture (see Table 6.6).

During the course of this investigation, the following observations were made. A high auxin : cytokinin ratio (2.0 : $2.0mgl^{-1}$) in combination with high light and low temperature (HL/LT) and low light and high temperature (LL/HT) conditions encouraged callusing of both donor and recipient tissue. Where the medium lacked both auxin and cytokinin, there was a tendency for browning to occur in the donor and recipient tissues. At a relatively low auxin : cytokinin ratio (0.2 : $0.2mgl^{-1}$) with both light and temperature regimes, no obvious pattern was exhibited. Where the auxin : cytokinin ratio was 10 : 1 and 1 : 0 (mgl^{-1}), swelling and curling of the donor tissue was noted, an effect more evident at low light and high temperature than at high light and low temperature. Browning and curling of the donor tissue was observed when the

medium was lacking in auxin but contained cytokinin (0 : 1 and 0 : 10mgl^{-1}). It was further observed, where the tetraploid species was the recipient tissue and the diploid species the donor tissue, regeneration of roots and shoots occurred more readily than with the reverse situation.

After four weeks of culture, 3% of heterografts had established firm graft unions (see Table 6.6). 58% of these were under low light/high temperature conditions and 42% under high light/low temperature conditions. Of the successful heterografts, 67% involved diploid tissue as the donor and tetraploid tissue as the recipient.

Key:

Media: MS + 3% sucrose (w/v) with combinations of NAA and BAP as detailed in Table 6.6

Temperature: 22 ± 1°C (LT); 27 ± 1°C (HT)

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR (HL); 27$\mu Mm^{-2}s^{-1}$PAR (LL)

Number of replicates per treatment: 12

Type of graft: heterograft

Period of culture: 28 days

2n → 4n: diploid tissue grafted onto tetraploid tissue

4n → 2n: tetraploid tissue grafted onto diploid tissue

Species and cultivars used: S. tuberosum ssp tuberosum cv. Pentland Ivory and cv. Majestic; S. sparsipilum

TABLE 6.6. Effect of NAA and kinetin on graft development and formation in thin cell layer heterografts of potato species

TYPE OF GRAFT	GROWTH REGULATOR CONCN. IN MEDIA (MGL^{-1})		LIGHT/TEMPERATURE = LL/HT			LIGHT/TEMPERATURE = HL/LT		
	NAA	KINETIN	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS	% GRAFT ESTABLISH-MENT	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS	% GRAFT ESTABLISH-MENT
2n→4n	0	0	58	42	-	75	75	-
		0.2	33	100	-	42	75	-
		2.0	67	75	33	50	58	-
4n→2n	0	0	8	17	-	33	33	-
		0.2	17	67	-	33	83	-
		2.0	-	-	25	42	50	8
2n→4n	0.2	0	75	100	-	67	100	-
		0.2	50	100	-	25	100	-
		2.0	33	67	-	58	100	-
4n→2n	0.2	0	25	100	-	25	75	-
		0.2	-	33	-	17	83	-
		2.0	-	-	-	8	100	-
2n→4n	2.0	0	-	25	-	-	67	-
		0.2	-	-	-	17	100	33
		2.0	-	25	-	33	100	-
4n→2n	2.0	0	-	100	-	-	100	-
		0.2	-	33	-	17	100	-
		2.0	-	-	-	17	100	-

6.2.8. Effect of 2,4-D and kinetin on graft development and formation

Culture technique and conditions were as described in Section 6.2.7, except that NAA in the culture medium was replaced by 2,4-D.

Similar observations were made as when NAA and kinetin were the growth regulators used, however, some differences in response were noted. Swelling and curling of the donor tissue occurred where the auxin : cytokinin ratio was 10 : 1 and 1 : 0 (mgl^{-1}) but with the latter ratio, the effect was more apparent under high light and low temperature (79%) than under the low light and high temperature (37%); this contrasted with the pattern observed when NAA was the auxin in the medium.

Of the heterografts cultured, 4% were successful (see Table 6.7). Of this 4%, 33% were cultured under high light and low temperature, 67% under low light and high temperature. Of the successful heterografts, 55% comprised diploid donor tissue grafted onto tetraploid recipient tissue.

Key:

Media: MS + 3% sucrose (w/v) with combinations of 2,4-D and kinetin as detailed in Table 6.7

Temperature: 22 ± 1°C (LT); 27 ± 1°C (HT)

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR (HL); 27$\mu Mm^{-2}s^{-1}$PAR (LL)

Number of replicates per treatment: 12

Period of culture: 28 days

Type of graft: heterograft

2n → 4n: diploid donor tissue grafted onto tetraploid recipient tissue

4n → 2n: tetraploid donor tissue grafted onto diploid recipient tissue

Species and cultivars used: S. tuberosum ssp tuberosum cv. Pentland Ivory and cv. Majestic; S. sparsipilum

TABLE 6.7. Effect of 2,4-D and kinetin on graft development and formation in thin cell layer heterografts of potato species

TYPE OF GRAFT	GROWTH REGULATOR CONCN^{-1} IN MEDIA (MGL^{-1})		LIGHT/TEMPERATURE = LL/HT			LIGHT/TEMPERATURE =HL/LT		
	2,4-D	KINETIN	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS	% GRAFT ESTABLISH-MENT	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS	% GRAFT ESTABLISH-MENT
2n→4n	0	0	42	58	-	25	42	-
		0.2	58	42	17	67	83	-
		2.0	58	75	-	42	42	-
4n→2n	0	0	25	25	-	33	42	-
		0.2	42	42	33	33	50	-
		2.0	25	42	-	-	50	-
2n→4n	0.2	0	83	100	-	-	100	-
		0.2	67	33	33	8	17	-
		2.0	-	62	-	25	100	-
4n→2n	0.2	0	42	75	-	-	100	-
		0.2	8	25	17	8	17	-
		2.0	-	-	-	25	100	-
2n→4n	2.0	0	-	75	-	17	92	-
		0.2	17	100	-	25	100	17
		2.0	-	10	-	-	33	17
4n→2n	2.0	0	-	-	-	-	-	-
		0.2	-	-	-	-	-	17
		2.0	-	-	-	-	33	-

6.2.9. Graft response on media containing 2,4-D and BAP

All procedures and conditions were as described in Section 6.2.8 except that BAP was substituted for kinetin in the culture medium. Responses to growth regulators were noted as in previous experiments (results are presented in Table 6.8).

In summary, where the auxin level is greater than the cytokinin level, swelling and expansion of the donor tissue was noted under both light and temperature conditions. Where the cytokinin level exceeded the auxin level, swelling of the donor tissue rarely took place under both light and temperature conditions. Browning of the donor tissue was seen more readily when the cytokinin level in the medium exceeded the auxin level. Expansion of the donor tissue was favoured by equal concentrations of auxin and cytokinin in the medium and low light/high temperature conditions, whereas under high light and low temperature, the greater proportion of donor tissue remained flat and did not expand. Where the auxin level in the medium either exceeded or was equal to the cytokinin level, callusing of the whole explant took place. However, where the auxin level was less than the cytokinin level in the medium, callus developed only at the ends of the explant.

12% of heterografts cultured during this experiment were successful. 62% of these were incubated under high light and low temperature conditions and 38% under low light and high temperature conditions. Of those successful grafts, 68% involved tetraploid tissue grafted onto diploid tissue (see Table 6.8). Grafting success was apparently favoured by high levels of auxin ($2.0 mgl^{-1}$) in combination with either $0.2 mgl^{-1}$ or $2.0 mgl^{-1}$ cytokinin.

Key:

Media: MS + 3% sucrose (w/v) with combinations of 2,4-D and BAP as detailed in Table 6.8

Temperature: 22 ± 1°C (LT); 27 ± 1°C (HT)

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR (HL);
27$\mu Mm^{-2}s^{-1}$PAR (LL)

Number of replicates per treatment: 12

Period of culture: 28 days

Type of graft: heterograft

2n → 4n: diploid tissue grafted onto tetraploid tissue

4n → 2n: tetraploid tissue grafted onto diploid tissue

Species and cultivars used: S. tuberosum ssp tuberosum cv. Pentland Ivory and cv. Majestic; S. sparsipilum

TABLE 6.8. Effect of 2,4-D and BAP on graft formation in thin cell layer heterografts of potato species

TYPE OF GRAFT	GROWTH REGULATOR CONCN. IN MEDIA (MGL^{-1})		LIGHT/TEMPERATURE = LL/HT			LIGHT/TEMPERATURE = HL/LT		
	2,4-D	BAP	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS	% GRAFT ESTABLISH-MENT	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS	% GRAFT ESTABLISH-MENT
2n→4n	0	0	-	-	-	-	-	-
		0.2	58	75	-	67	75	-
		2.0	25	8	-	8	17	-
4n→2n	0	0	-	-	-	-	-	-
		0.2	17	58	50	67	67	-
		2.0	70	-	-	50	42	-
2n→4n	0.2	0	83	100	-	25	75	-
		0.2	33	17	-	58	75	-
		2.0	42	58	-	58	58	8
4n→2n	0.2	0	25	58	-	8	75	-
		0.2	17	42	-	17	58	50
		2.0	-	17	8	25	42	17
2n→4n	2.0	0	-	75	-	25	83	-
		0.2	18	100	58	8	100	33
		2.0	17	75	8	17	50	25
4n→2n	2.0	0	8	83	-	-	42	-
		0.2	-	33	17	-	100	58
		2.0	-	-	17	17	42	67

6.2.10. Graft response on media containing NAA and BAP

All procedures and conditions were as described in Section 6.2.9 except that NAA was substituted for 2,4-D in the culture medium. Lower levels of growth regulators ($0.02mgl^{-1}$) were used than in previous experiments with the aim of reducing callus formation and then evaluating the effect such a reduction would have on the graft union between donor and recipient tissue.

The species and cultivars used in this experiment were S. tuberosum ssp tuberosum cv. Pentland Ivory and S. sparsipilum. For each medium combination and light and temperature treatment, there were 16 heterografts. Heterografts were also cultured as controls on basal medium lacking in growth regulators (Section 2.2). Results were assessed after heterografts had been cultured for 14 and 30 days (Table 6.9).

After 14 days of culture it was difficult to establish the extent of cohesion between donor and recipient tissue from mere observation. The extent of cohesion could not be determined by attempting to remove the donor tissue from the recipient tissue using forceps as it was felt that this would be likely to interfere with the natural development of the graft. Explants had produced roots when cultured on media containing auxin equal to, or in excess of, cytokinin level. Roots were initiated from the recipient tissue, and tended to be more prolific with a tetraploid recipient tissue than with a diploid recipient tissue.

The maximum grafting success (50%) occurred when explants were cultured on media containing 2.0NAA and either 0.2BAP or 0.02

BAP mgl^{-1} (Table 6.9). Under both light and temperature regimes, the majority of successful grafts were cultured on media containing $2mgl^{-1}$NAA, and few or no grafts succeeded when there was no auxin or cytokinin in the medium. Of all the grafts cultured under the lower light intensity ($27\mu Mm^{-2}s^{-1}$PAR) 4% were successful; under the higher light intensity ($85\mu Mm^{-2}s^{-1}$PAR), 13% were successful. Success rate was marginally higher when the graft involved tetraploid donor tissue and diploid recipient tissue (11%) rather than the reverse (6%). As found previously (Section 6.2.9), grafting success was favoured by high levels of auxin ($2mgl^{-1}$).

TABLE 6.9. Effect of NAA and BAP on graft development and formation in thin cell layer heterografts of potato species

GROWTH REGULATOR CONCENTRATION IN MEDIUM (MGL^{-1})		% GRAFT ESTABLISHMENT			
		HL/LT		LL/HT	
NAA	BAP	2n→4n	4n→2n	2n→4n	4n→2n
-	-	-	-	-	-
-	0.02	-	-	-	-
-	0.2	-	25	-	-
-	2.0	-	-	-	12
0.02	-	-	37	-	12
0.02	0.02	12	25	-	-
0.02	0.2	12	37	-	-
0.02	2.0	-	12	-	-
0.2	-	-	-	-	-
0.2	0.02	-	-	-	-
0.2	0.2	12	-	-	-
0.2	2.0	-	12	-	-
2.0	-	-	-	-	-
2.0	0.02	50	37	12	50
2.0	0.2	50	12	12	12
2.0	2.0	37	37	-	12

Key:

Media: MS + sucrose (w/v) with combinations of NAA and BAP as detailed in Table 6.9

Temperature: 22 ± 1°C (LT); 27 ± 1°C (HT)

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR (HL);
27$\mu Mm^{-2}s^{-1}$PAR (LL)

Number of replicates per treatment: 16

Period of culture: 30 days

Species and cultivars used: S. tuberosum ssp tuberosum cv. Pentland Ivory; S. sparsipilum

Type of graft: heterograft

2n → 4n: diploid tissue grafted onto tetraploid tissue

4n → 2n: tetraploid tissue grafted onto diploid tissue

6.2.11. Importance of close tissue contact on graft development

This investigation had a three-fold aim: primarily, it was intended that the importance of securing thin cell layers (donor tissue) onto stem sections (recipient tissue) should be ascertained; secondly, its purpose was to confirm results obtained in Section 6.2.10, and finally, the role of light intensity and temperature was further considered by employing different regimes from those described in Section 6.2.10.

Culture techniques and conditions were as described in Section 6.2.2, and sterile P.T.F.E. tape was used to secure contact between donor and recipient tissue. P.T.F.E. tape was chosen because it was relatively easy to work with under in vitro conditions, and could be sterilised in an autoclave without undergoing any apparent change in structure or form. Sections of P.T.F.E. tape (already sterilised, see Section 2.4), were cut into strips of sufficient length to wrap around the complete circumference of the grafted explant, that is, donor and recipient tissue. Because of the relative adhesiveness of the tape, administering slight pressure to both ends of the tape once in contact with each other guaranteed the tape would remain in place, thus ensuring that the donor and recipient tissues remained in contact during the early stages of graft development.

Basal culture medium (Section 2.2) was supplemented with 3% sucrose (w/v) and with NAA and BAP in the following combinations: $2.0/0.02 mgl^{-1}$; $2.0/0.2 mgl^{-1}$ and $2.0/2.0 mgl^{-1}$. This choice of growth regulators was based on the results as shown in Section 6.2.10

(Table 6.9). Heterografts were cultured under a total of five different light and temperature regimes (Section 2.3). The species and cultivars used in this investigation were as noted in Section 6.2.10. For each medium combination, and light and temperature treatment, there were 20 heterografts. Results were assessed after 14 and 30 days of culture.

The stages of development followed by the grafted explants are shown by Plates 6.1A to 6.1I. After three days of culture both types of graft (2n → 4n, that is, diploid donor tissue grafted onto tetraploid recipient tissue, and 4n → 2n, that is, tetraploid donor tissue grafted onto diploid recipient tissue) were exhibiting similar levels of expansion, in that a minimal degree of expansion had been undergone by the recipient tissue (Plate 6.1B). A further three days of culture revealed expansion of both donor and recipient tissue, splitting of the P.T.F.E. tape due to this expansion and the initiation of callus production at both ends of the recipient tissue (Plate 6.1C). By day nine, the amount of expansion and callus formation had increased (Plate 6.1D). Prior to day 12, there were no obvious differences in the two types of graft, however, around day 12, the rate and type of callus production was beginning to distinguish the two types of grafts. With 4n → 2n grafts, expansion and callus production had extended to the sides of the recipient tissue, whereas with 2n → 4n grafts development was seemingly confined to the ends of the recipient tissue (Plate 6.1E and 6.1F). In addition, swelling of the donor tissue had increased with both types of grafts, but was more advanced with 4n → 2n grafts than 2n → 4n. At this stage, cell division was observed in the area

between the donor tissue and the recipient tissue, however, it was not easy to ascertain whether this was a response expressed by both partners of the graft or merely one. This pattern of development continued, with callus production by 4n → 2n graft exceeding that of the 2n → 4n graft. Furthermore, the tetraploid donor tissue continued to expand, and in some grafts this was seen to result in diminished contact between the partners. By day 21, extensive production of white spongy callus had occurred with the 4n → 2n graft, and in some cases this callus had extended over a major portion of the grafted explant (Plate 6.1G). After 28 days of culture, this type of development had proceeded further: the donor tissue in most 4n → 2n grafts was only partially visible, but could be seen to be very swollen and green in colour (Plate 6.1H). Conversely with 2n → 4n grafts, expansion and callus production had remained relatively limited. However, the diploid donor tissue possessed the same green and swollen appearance, though the latter was apparent to a lesser extent (Plate 6.1I). Close observation of these grafts revealed that cell division had occurred between the two partners.

Root development occurred and was favoured by light intensities greater than $27\mu Mm^{-2}s^{-1}$PAR. Cohesive strength between donor and recipient tissue was encouraged by the presence of $2.0mgl^{-1}$ NAA in the medium (Table 6.1O), and with the exception of results achieved under a light intensity of $300\mu Mm^{-2}s^{-1}$PAR, the number of successful grafts was greater when the media contained levels of cytokinin in excess of $0.02mgl^{-1}$. As Table 6.1O shows maximum graft establishment (60%) was obtained at a temperature of $22^{o}C$, a light intensity of $85\mu Mm^{-2}s^{-1}$PAR and a 16h daylength (HL/LT) on a medium

containing 2.0NAA and 2.0BAP (mgl^{-1}). The results of this experiment tend to imply that close tissue contact achieved through using PTFE tape is an important factor in the process of maximising graft establishment. Prior to using PTFE tape the maximum graft establishment obtained was 50% (see Table 6.9). Although the 60% graft establishment achieved in this experiment is not a substantial increase on this previous maximum, a further comparison of other results achieved in Section 6.2.10 (Table 6.9) with the results from this experiment (Table 6.10) reveals a general increase in the percentage graft establishment on the implementation of PTFE tape in the grafting technique.

Key:

Media: MS + 3% sucrose (w/v) with combinations of NAA and BAP as detailed in Table 6.10.

Number of replicates per treatment: 20

Period of culture: 30 days

Type of graft: heterograft

2n → 4n: diploid donor tissue grafted onto tetraploid tissue

4n → 2n: tetraploid tissue grafted onto donor tissue

Species and cultivars used: S. tuberosum ssp. tuberosum cv. Pentland Ivory; S. sparsipilum

Light and temperature conditions:

LIGHT AND TEMPERATURE CONDITIONS	TEMPERATURE (^{o}C)	DAYLENGTH (HOURS)	LIGHT INTENSITY ($\mu Mm^{-2}s^{-1}$ PAR)
LL/LT(8)	22 ± 1	8	5
LL/LT(16)	22 ± 1	16	20
HL/LT	22 ± 1	16	85
LL/HT	27 ± 1	continuous	27
HL/HT	27 ± 1	continuous	300

TABLE 6.10. Importance of close tissue contact on percentage graft establishment

TYPE OF GRAFT	LIGHT AND TEMPERATURE CONDITIONS					MEDIA (MGL^{-1})
	LL/LT (8)	LL/LT (16)	HL/LT	LL/HT	HL/HT	
2n→4n	70	CONTAMINATED	40	10	40	2.ONAA + 0.02BAP
4n→2n	-		10	30	40	
MEAN GRAFT ESTABLISHMENT	35		25	20	40	
2n→4n	30	30	50	20	40	2.ONAA + 0.2BAP
4n→2n	50	50	60	40	40	
MEAN GRAFT ESTABLISHMENT	40	40	55	30	40	
2n→4n	30	CONTAMINATED	60	40	50	2.ONAA + 2.OBAP
4n→2n	40		60	50	30	
MEAN GRAFT ESTABLISHMENT	35		60	45	40	

Plate 6.I. Development of a thin cell layer heterograft between *S. sparsipilum* (diploid) and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory on a medium containing NAA ($2mgl^{-1}$) and BAP ($2mgl^{-1}$)

A. A thin cell layer heterograft composed of diploid donor tissue (A) grafted onto tetraploid recipient tissue at the time of construction i.e. day zero x 16

B. As Plate 6.IA but after three days of culture; recipient tissue has expanded slightly x 16

C. As Plate 6.IA but after six days of culture; expansion of both donor and recipient tissue has resulted in the splitting of the PTFE tape (A). The initiation of callus production can be seen at both ends of the recipient tissue (B) x 16

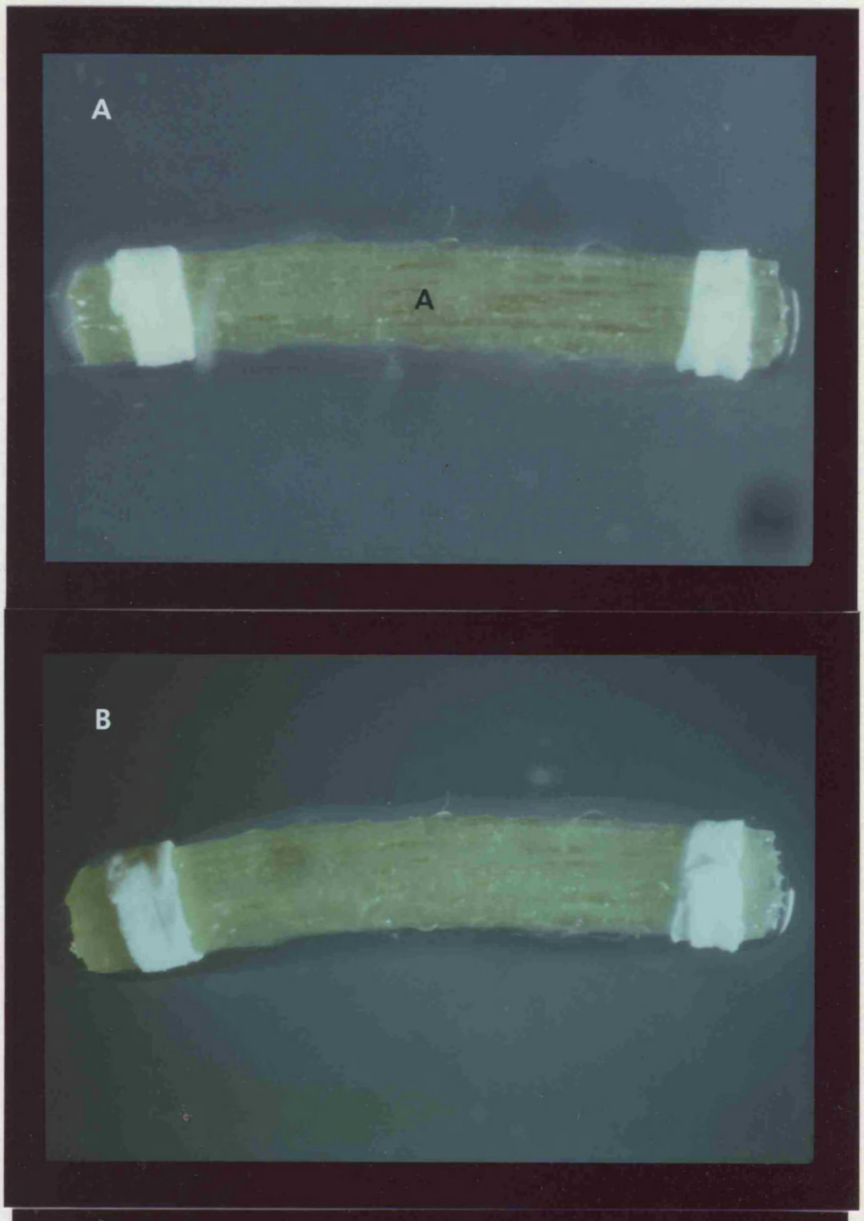

A
A
B
C
A
B
A
B

Plate 6.I. Development of a thin cell layer heterograft between *S. sparsipilum* (diploid) and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory (tetraploid) on a medium containing NAA ($2mgl^{-1}$) and BAP ($2mgl^{-1}$)

D. As Plate 6.IA but after nine days of culture; tissue expansion and callus production as described for Plate 6.IC has increased x 25

E. A thin cell layer heterograft composed of tetraploid donor tissue grafted onto diploid recipient tissue after 12 days of culture; callus production has increased and has extended to the sides of the recipient tissue (A) x 16

F. A thin cell layer heterograft composed of diploid donor tissue grafted onto tetraploid recipient tissue after 12 days of culture; callus production is confined to the ends of the recipient tissue (A) x 16

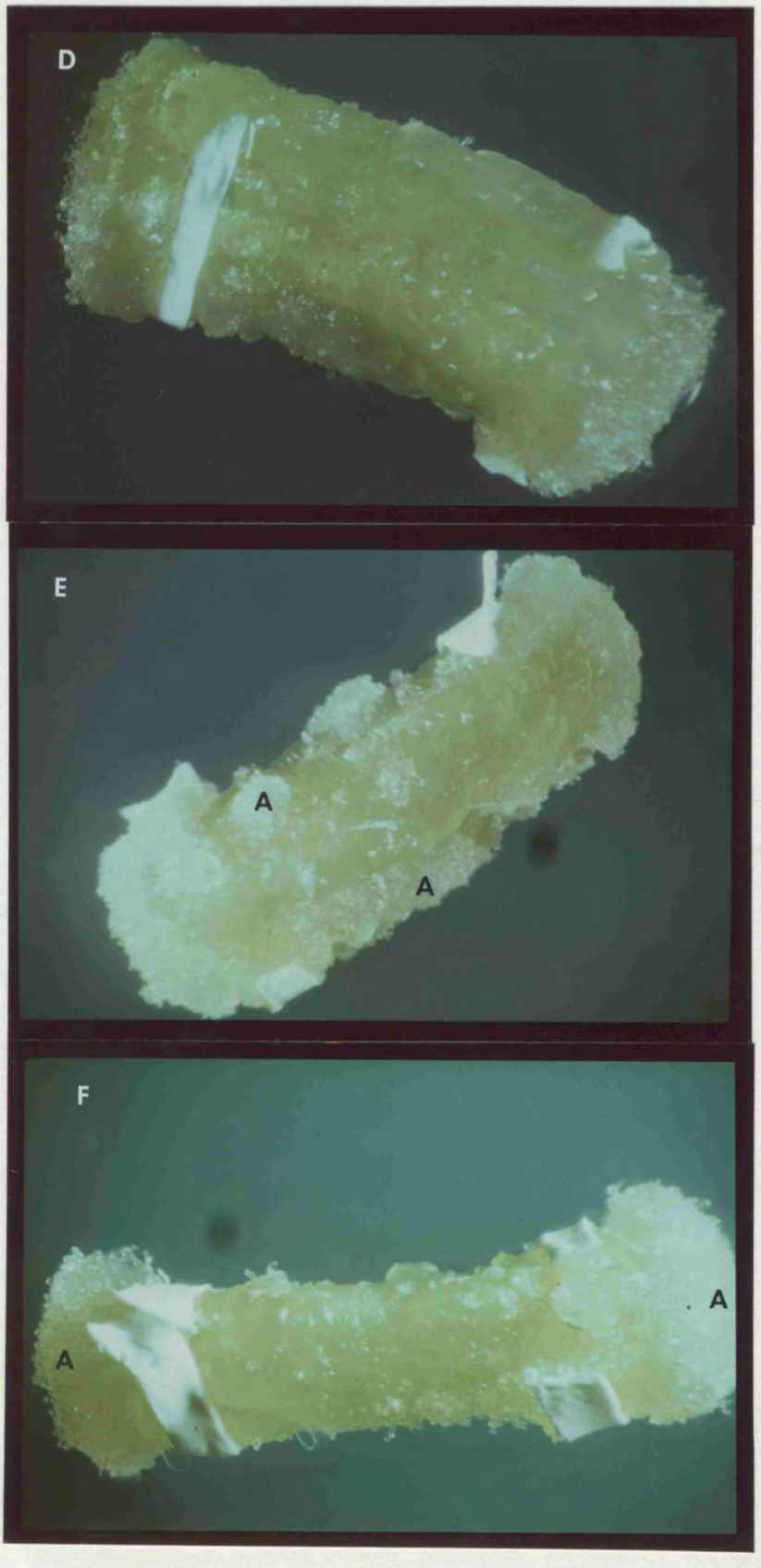
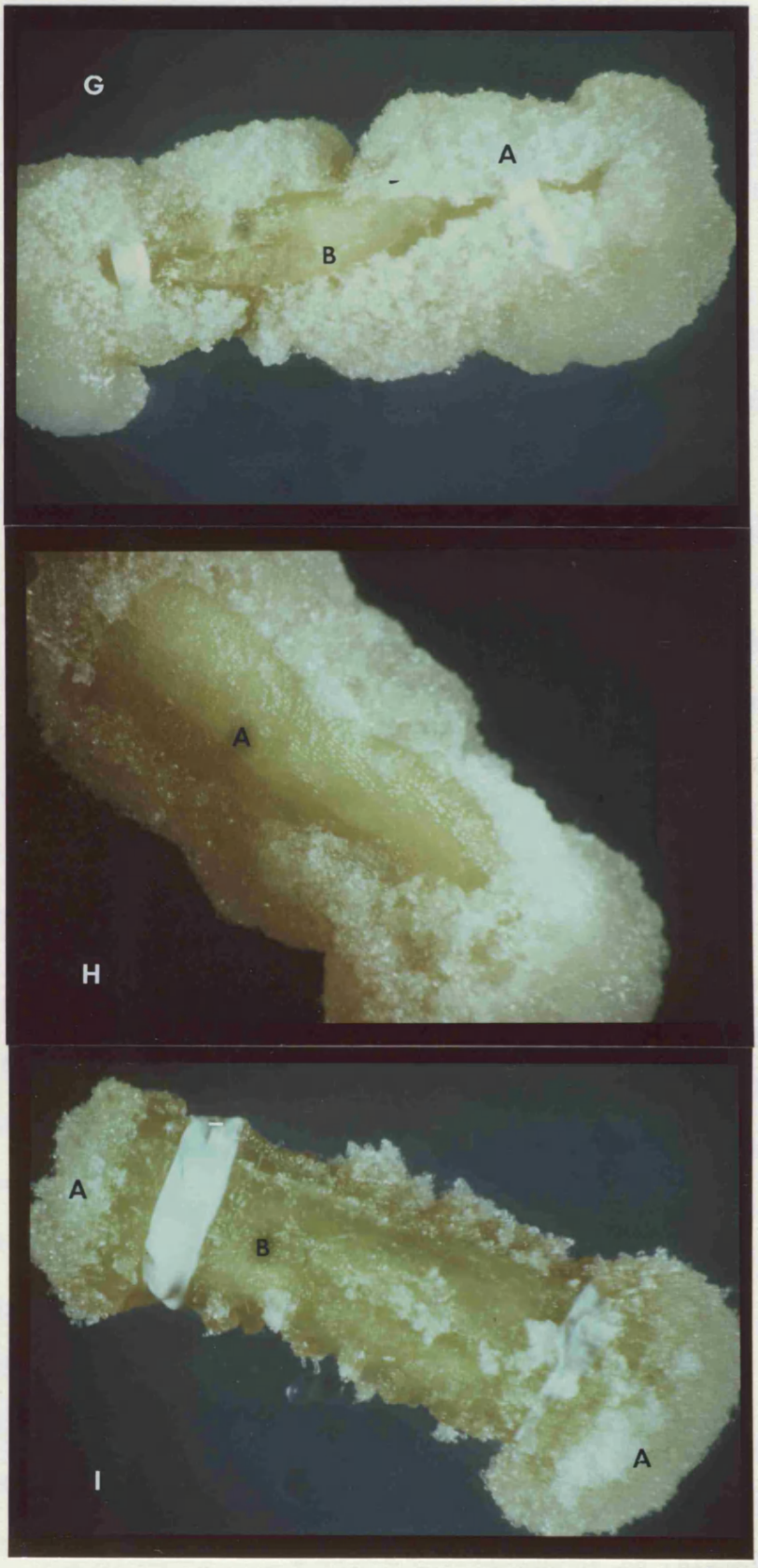

D
E
A
A
F
A
A

Plate 6.I. Development of a thin cell layer heterograft between S. sparsipilum (diploid) and S. tuberosum ssp. tuberosum cv. Pentland Ivory on a medium containing NAA ($2mgl^{-1}$) and BAP ($2mgl^{-1}$)

G. As Plate 6.IE but after 21 days of culture. Extensive production of white spongy callus (A) has occurred covering a major portion of the grafted explant. Some of the donor tissue (B) is still visible x 16

H. As Plate 6.IG but after 28 days of culture. The donor tissue is swollen and slightly green in colour (A) x 25

I. A thin cell layer heterograft composed of diploid donor tissue grafted onto tetraploid recipient tissue after 28 days of culture. Expansion and callus production has remained relatively limited, and mainly confined to the ends of the explant (A). The diploid donor tissue is slightly swollen and green in colour (B) x 16


G
A
B
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I

6.2.12. Cultivar effect on graft union

The aim of this investigation was to obtain some indication of the general applicability of this grafting system by replacing the tetraploid partner with another tetraploid cultivar.

Culture technique and conditions were as detailed in Section 6.2.11. Media and light and temperature treatments were selected based on results outlined in Section 6.2.11. The replacement tetraploid partner chosen was S. tuberosum ssp. tuberosum cv. Fortyfold. S. sparsipilum remained as the diploid partner because of its good regenerative potential (Section 7.2) and the comparative ease with which thin cell layers could be excised. For each medium combination and light and temperature treatment, there were 36 heterografts. Heterografts were also cultured on basal culture medium (Section 2.2), without growth regulators. Results were assessed after 14 and 30 days of culture (Table 6.11).

After 14 days of culture, there was extensive browning of the donor tissue of the heterografts cultured on media lacking in growth regulators. Graft establishment was assessed after 30 days of culture and as Table 6.11 shows maximum graft establishment was obtained when cv. Fortyfold was the tetraploid partner (83%). These results further show that the culture conditions indicated in Section 6.2.11 as favouring graft formation, that is, 2.0NAA and 2.0BAP (mgl^{-1}) in the medium; 22°C temperature; a light intensity of 85$\mu Mm^{-2}s^{-1}$PAR and a daylength of 16h; close tissue contact, has resulted in maximising graft establishment in this experiment also. The percentage graft establishment obtained during this experiment when cv. Pentland Ivory was the tetraploid partner is

similar to those results obtained in Section 6.2.11 (Table 6.10), and thus gives some indication as to the predictability of this grafting technique. As Table 6.11 shows, there was also evidence from this experiment that grafts where the donor tissue is of diploid origin and the recipient tissue is of tetraploid origin occur more readily than in the reverse situation.

TABLE 6.11. Cultivar effect on percentage graft establishment

TYPE OF GRAFT	MEDIA AND LIGHT TEMPERATURE TREATMENT				
	HL/LT ON/OB	HL/LT 2.ON/O.2B	HL/LT 2.ON/2.OB	LL/HT ON/OB	LL/HT 2.ON/2.OB
cv Fortyfold as 4n partner					
2n→4n	0	66	83	0	50
4n→2n	0	44	83	0	44
MEAN GRAFT ESTABLISHMENT	0	55	83	0	47
cv Pentland Ivory as 4n partner					
2n→4n	0	55	61	0	55
4n→2n	0	50	55	0	33
MEAN GRAFT ESTABLISHMENT	0	53	58	0	44

Key:

Media: MS + 3% sucrose (w/v) with combinations of NAA and BAP (mgl^{-1}) as detailed above

Number of replicates per treatment: 36

Period of culture: 30 days

Type of graft: heterograft

2n → 4n: diploid tissue grafted onto tetraploid tissue

4n → 2n: tetraploid tissue grafted onto diploid tissue

Species and cultivars used: *S. tuberosum* ssp. *tuberosum* cv. Fortyfold, cv. Pentland Ivory; *S. sparsipilum*

Temperature: 22 ± 1°C; 27 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR (HL/LT)

6.2.13. The effect of NAA and BAP grafting medium on the development of a union between tissues of the same genotype

Culture techniques and conditions were as detailed in Section 6.2.12 with the omission of explants cultured under low light intensity ($27\mu Mm^{-2}S^{-1}$PAR) and the culture medium containing 2.0NAA and 0.2BAP (mgl^{-1}). Autografts of S. tuberosum ssp. tuberosum cv. Pentland Ivory and S. sparsipilum were assembled, in addition, heterografts between these two species were also constructed.

The pattern of development followed that described in Section 6.2.11 and a comparison of Table 6.12 with Table 6.10 (Section 6.2.11) shows that the percentage graft establishment for heterografts was similar in both experiments. In addition, the results of this experiment indicated that the diploid species are more receptive to the grafting procedure than the tetraploid species. As Table 6.12 shows, when the tissue involved in the autograft originated from the same diploid source, that is, S. sparsipilum, percentage graft establishment was 67%. However, when considering autografts comprised of tetraploid tissue, that is, S. tuberosum cv. Pentland Ivory, grafting success was 44%.

TABLE 6.12. The effect of NAA and BAP grafting medium on the development of a graft union between tissues of the same genotype

GRAFT TYPE	% GRAFT ESTABLISHMENT
S. sparsipilum (autograft)	67
S. tuberosum ssp. *tuberosum* cv. Pentland Ivory (autograft)	44
Diploid tissue grafted on to tetraploid tissue (2n → 4n)	64
Tetraploid tissue grafted on to diploid tissue (4n → 2n)	55

Key:

Media: MS + 3% sucrose (w/v) with combinations of NAA and BAP (2.0NAA + 2.0BAP mgl^{-1})

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Number of replicates per treatment: 36

Period of culture: 40 days

2n → 4n: diploid tissue grafted onto tetraploid tissue

4n → 2n: tetraploid tissue grafted onto diploid tissue

6.2.14. Effect of inclusion of GA_3 in the culture medium

The aim of this experiment was to assess the influence, that the presence of GA_3 in the grafting medium would have on the number of successful grafts and, subsequently, on the regeneration of those grafts. It has been shown by Roberts et al., (1965) that GA_3 reduces the extent of callus formation between graft partners without lessening graft union strength. Such a reduction in the volume of callus might facilitate chimera production in that tissues of different genotypes would be comparatively closer together. Furthermore, it has been demonstrated that the addition of GA_3 to the culture medium has favoured shoot development and elongation in potato (Roest et al., 1976; Webb et al., 1983).

Culture technique and conditions were as described in Section 6.2.11. Basal culture medium (Section 2.2) was supplemented with 3% sucrose (w/v) and the following combinations of growth regulators: TABLE 6.13

GROWTH REGULATORS IN MGL^{-1}			
MEDIA	NAA	BAP	GA_3
1	2.0	2.0	-
2	2.0	2.0	0.5
3	2.0	2.0	1.0

Heterografts were cultured under a 16h daylength of 85$\mu Mm^{-2}s^{-1}$PAR at 22°C (HL/LT). The species and cultivars used in this investigation·

were noted in Section 6.2.10. For each medium combination, there were 30 heterografts.

Development of the heterografts in this experiment proceeded as described in Section 6.2.11, however, the expansion of donor tissue was markedly greater when the heterografts were cultured on media containing GA_3. In addition, the donor tissue acquired a translucent appearance, an effect not noted when the grafting medium lacked GA_3. After ten days of culture, roots had developed, root production being favoured by a medium where GA_3 was absent (Table 6.14). Similarly, grafting success was at its maximum when heterografts had been cultured on media lacking in GA_3. As Table 6.14 shows, the presence of GA_3 in the grafting medium appeared to impede the grafting process in some way, in that as the level of GA_3 in the medium increased, percentage grafting success decreased.

TABLE 6.14. Effect of GA_3 in grafting medium on heterograft development

GROWTH REGULATORS IN MEDIUM (mgl^{-1})	% GRAFT ESTABLISHMENT		% EXPLANTS WITH ROOTS
	2n → 4n	4n → 2n	
2.0NAA + 2.0BAP	87	73	27
2.0NAA + 2.0BAP + $0.5GA_3$	13	13	-
2.0NAA + 2.0BAP + $1.0GA_3$	6	13	13

Key:

Media: MS + 3% sucrose (w/v) with combinations of NAA and BAP as detailed above

Temperature: 22 ± 1°C

Light conditions: 16h daylength; $85\mu Mm^{-2}s^{-1}$ PAR

Number of replicates per treatment: 30

Period of culture: 21 days

2n → 4n: diploid tissue grafted onto tetraploid tissue

4n → 2n: tetraploid tissue grafted onto diploid tissue

Species and cultivars used: S. tuberosum ssp. tuberosum cv. Pentland Ivory; S. sparsipilum

6.2.15. Influence of age of tissue on graft formation

This investigation resulted from observations made in Section 6.2.14 showing that the grafting success achieved on the medium containing 2.0NAA and 2.0BAP (mgl^{-1}) had improved compared with results from Section 6.2.11. Furthermore, strong graft unions had been attained within 21 days of culture, seven days less than in previous grafting experiments. The only difference between the two experiments that was evident, was tissue age, in that, explants used in Section 6.2.14 were derived from shoot-tips that had been cultured *in vitro* for three weeks, unlike previous experiments where the tissue was derived from shoot-tips that had been cultured *in vitro* for four to six weeks.

Culture technique and conditions were as detailed in Section 6.2.14, except that only one medium was used, namely 2.0NAA + 2.0BAP mgl^{-1}. In addition, material that had been in culture for three weeks (plantlets derived from shoot-tips) was selected to be grafted, together with tissue that had been cultured for six weeks since initial excision of the shoot-tip. Thus, heterografts were either composed of three week old tissue or six week old tissue.

The results of this investigation confirmed what had been observed in the previous experiment (Section 6.2.14), namely that heterografts composed of three week old tissue developed faster than those composed of six week old tissue. This resulted in improved grafting success in terms of numbers of successful grafts achieved and period of time required for strong graft unions to form (Table 6.15).

TABLE 6.15. Influence of age of tissue on graft union formation

PERCENTAGE GRAFT ESTABLISHMENT			
THREE WEEK OLD TISSUE		SIX WEEK OLD TISSUE	
2n → 4n	4n → 2n	2n → 4n	4n → 2n
73	80	53	60

Key:

Media: MS + 3% sucrose (w/v) with combinations of NAA and BAP (2.0NAA + 2.0BAP mgl^{-1})

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Number of replicates per treatment: 30

Period of culture for three week old tissue: 21 days

Period of culture for six week old tissue: 30 days

2n → 4n: diploid tissue grafted onto tetraploid tissue

4n → 2n: tetraploid tissue grafted onto diploid tissue

Species and cultivars used: S. tuberosum ssp. tuberosum cv. Pentland Ivory; S. sparsipilum

6.2.16. Histology of grafted explants

Explants (heterografts) were prepared and cultured as described in Section 6.2.11, on basal culture medium supplemented with 3% sucrose (w/v) and 2.0NAA + 2.0BAP (mgl^{-1}). At various stages during the grafting process, grafted explants were taken out of culture and prepared for histological examination (Section 2.5). The purpose of this process was to confirm heterograft formation, and to elucidate the processes leading up to the formation of a graft union between donor and recipient tissue.

Observations from sections of explants cultured for 12 days after grafting revealed the presence of callus between the two graft partners. It would appear that callus production was initiated by the living cells lying immediately beneath the cut cells at the graft interface. Extension of these cells into the graft space between the donor and recipient tissue, followed by division, resulted in callus formation which was seemingly derived from both graft partners. This invasive growth of callus was seen to begin at the ends of the graft prior to any other site (Plate 6.2A). However, a further six days of culture showed that this intrusive growth of cells had filled most of the original gap between the donor and recipient tissue (Plate 6.2B). The callus cells which were produced, tended to be highly vacuolate with extremely thin walls. Thus, after approximately 18 days of culture, a callus bridge had been formed which extended throughout the length of the graft.

Later sections revealed differentiation occurring across the callus bridge, which appeared to be a form of wound xylem and phloem characterised by meandering strands of irregularly shaped

cells. This was observed in sections taken from explants in culture for 30 days. As a general rule, cell differentiation within the callus bridge was observed mainly in cells sited towards the ends of the graft (Plate 6.2C). However, some sections showed cell differentiation occurring at other points throughout the callus bridge (Plate 6.2D). The presence of tracheary-type elements was noted in many sections, within the callus bridge (Plate 6.2D) and/or the adjacent donor and recipient tissue. Increased starch formation was a response which was noted in many of the grafted explants examined. This activity tended to be confined within the donor tissue, however, in some instances, it was associated with the cells which were differentiating within the callus bridge (Plate 6.2F).

The ability of donor and recipient tissues to establish a union within a small area of the graft was illustrated by examination of some explants (Plate 6.2E); the remaining donor and recipient tissue remained undeveloped with respect to graft formation.

The majority of grafted explants sectioned revealed the presence of a callus bridge within which cells were undergoing differentiation. However, a few grafts were observed where the indications were that little or no callus had developed, but that donor tissue and recipient tissue were in direct contact except for the existence of a slightly darker staining area between the two tissues (Plate 6.2G). Closer examination revealed no apparent cell division within the rec ipient tissue. However, as can be seen by Plate 6.2G, this inactivity was not extended to the donor tissue, where a shoot structure had been initiated. Furthermore, vascular

differentiation (characterised by smaller, deeply staining cells) had occurred within the donor tissue, namely in the cells underlying those involved in shoot formation (Plate 6.2H), but this differentiation ceased within a few cells of the darker staining area. With reference to the latter, there was evidence that this layer had existed in many of the grafts examined, but it would seem that development of the callus bridge and cell differentiation within the callus bridge resulted in a rupturing of this layer and its subsequent displaeement.

Plate 6.2. Stages in the development of thin-cell layer heterografts between S. sparsipilum (A) and S. tuberosum ssp. tuberosum cv. Pentland Ivory (B) cultured on media containing NAA ($2.0 mgl^{-1}$) and BAP ($2.0 mgl^{-1}$). The heterografts consisted of the diploid tissue (A) grafted onto tetraploid tissue (B)

A. Callus development between the donor component (A) of a heterograft and the recipient component (B) after 12 days of culture. Callus initiation was observed at the ends of the graft prior to any other site. x 44

B. After 18 days of culture the donor component (A) of the heterograft and the recipient component (B) were seen to be connected by a callus bridge (C) which extended throughout the length of the graft. x 44

C. After 30 days of culture, differentiation (D) was observed within the cells of the callus bridge (C). The differentiation appeared to be a form of wound xylem and phloem characterised by meandering strands of irregularly shaped cells. x 109

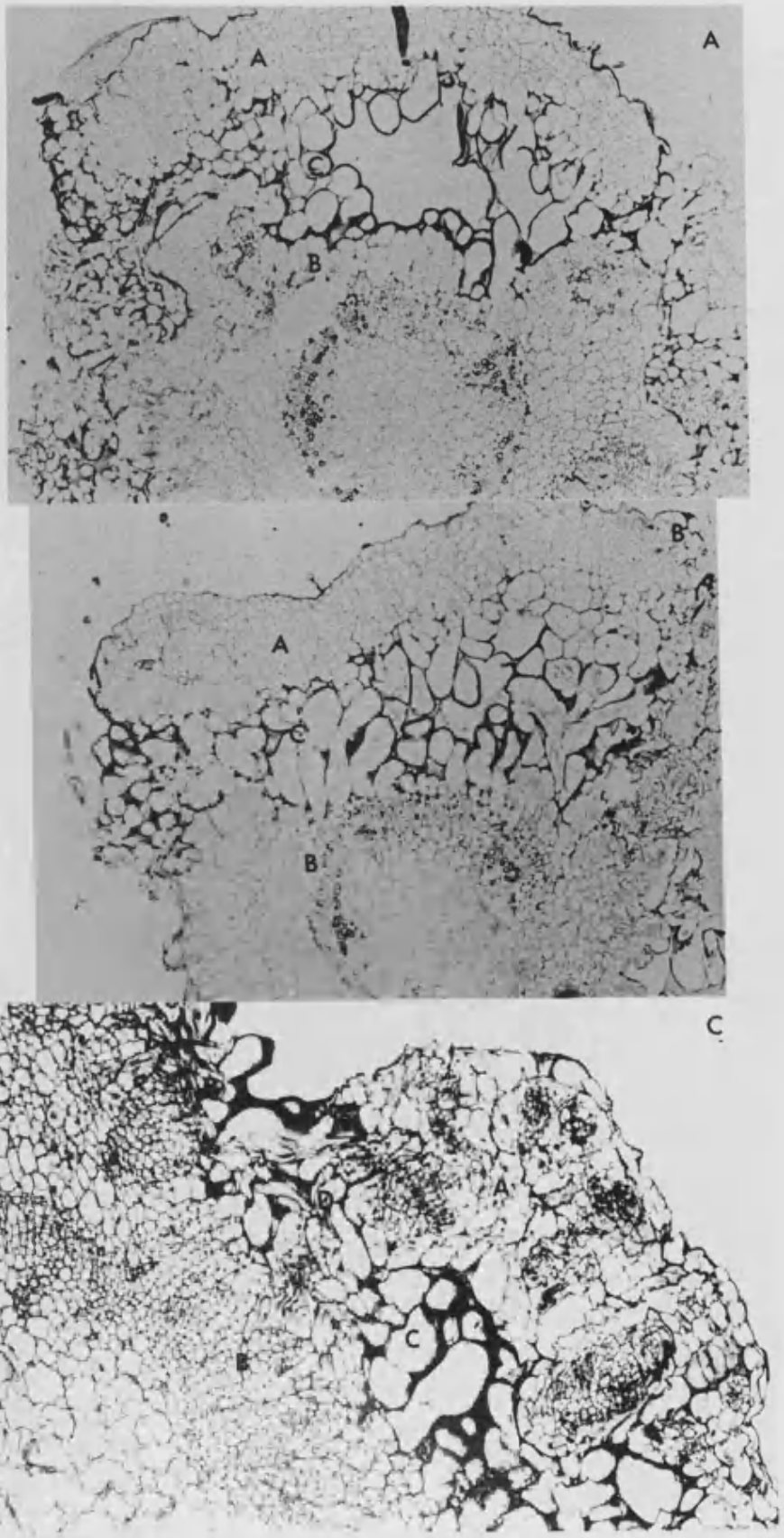

A
A
B
B
A
B
C
A
B
C

Plate 6.2. Stages in the development of thin-cell layer heterografts between S. sparsipilum (A) and S. tuberosum ssp tuberosum cv. Pentland Ivory (B) cultured on media containing NAA ($2.0 mgl^{-1}$) and BAP ($2.0 mgl^{-1}$). The heterografts consisted of diploid tissue (A) grafted onto tetraploid tissue (B)

D. Tracheary-type elements (D) within the callus bridge (C) separating the donor (A) and recipient (B) tissues of the heterograft x 109

E. Donor (A) and recipient (B) tissues connected by a small bridge of cells x 109

F. Starch activity observed within the cells of the callus bridge (c) both within vacuolated cells (E) and in association with cells undergoing differentiation (D) x 219

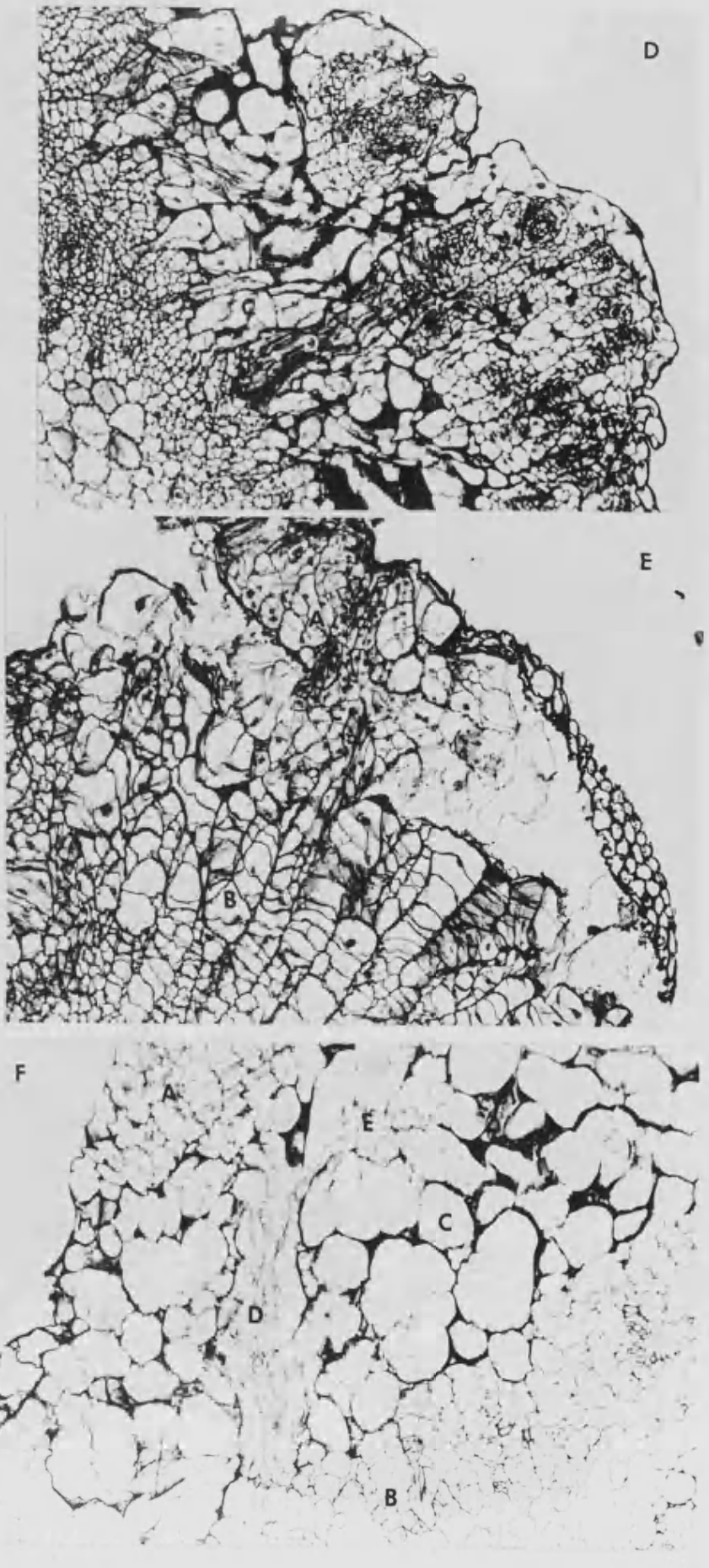
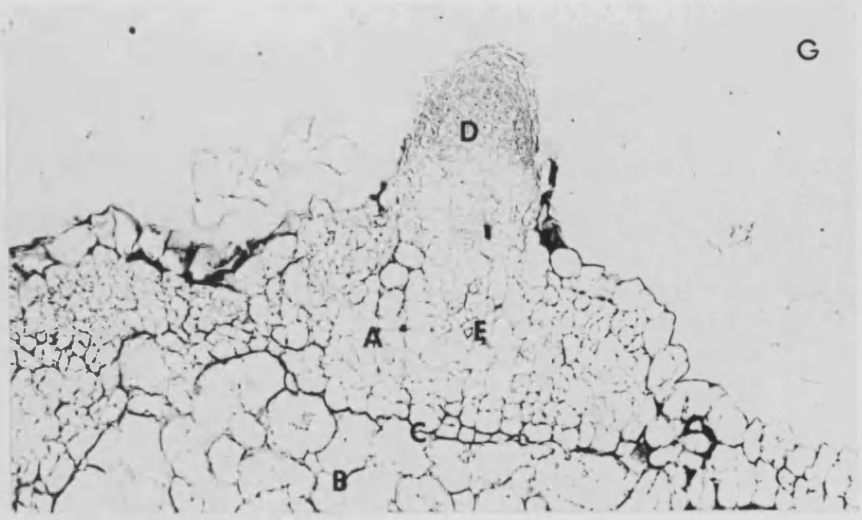

D
C
E
A
B
F
A
E
C
D
B

Plate 6.2. Stages in the development of thin-cell layer heterografts between S. sparsipilum (A) and S. tuberosum ssp. tuberosum cv. Pentland Ivory (B) cultured on media containing NAA ($2.0 mgl^{-1}$) and BAP ($2.0 mgl^{-1}$). The heterografts consisted of diploid tissue (A) grafted onto tetraploid tissue (B)

G. Shoot initiation (D) from the donor tissue (A) of the hetero-graft. This section shows donor tissue (A) and recipient tissue (B) in direct contact except for the existence of a slightly darker staining area between the two tissues (C). Vascular differentiation characterised by smaller, deeply staining cells (E) had occurred within the donor tissue (A). x 109

H. As G but showing the area of vascular differentiation (D) within the donor tissue in greater detail. x 219

I. The presence of a darker staining layer (D) was observed in some heterografts on the donor side of the callus bridge (C). It would appear that this layer was ruptured and thus penetrated by the cells differentiating within the callus bridge. x 109


G
D
A
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C
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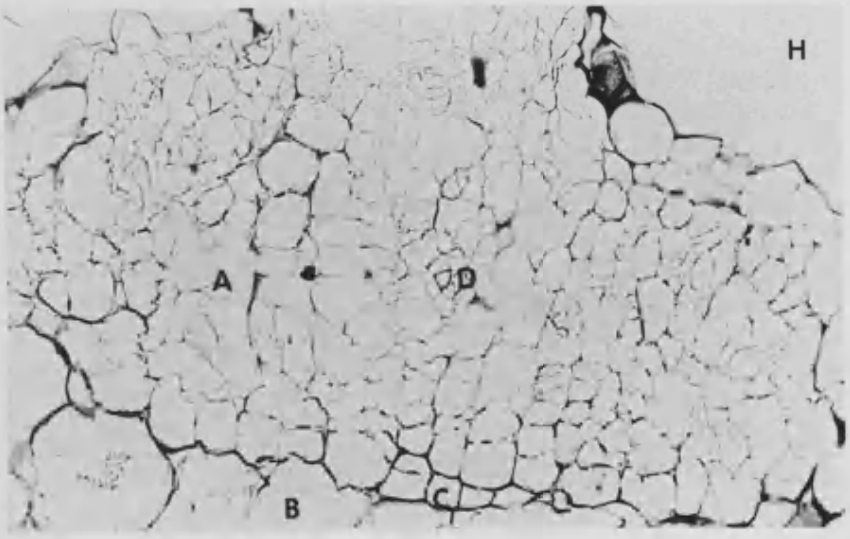
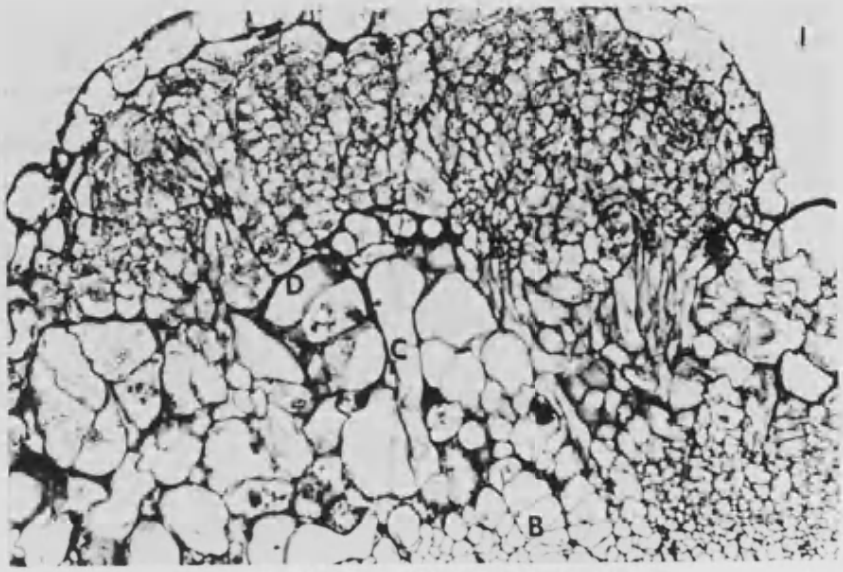

H
A
D
B
C


I
D
C
B

6.2.17. Discussion

Unsuccessful grafts *in vivo* can result from mechanical mismatches in size or tissue location, desiccation of tissues, viral infections and adverse temperature or light regimes (Roberts, 1949; Rogers *et al.*, 1957; Hartmann *et al.*, 1975). Further factors require consideration once grafts are established *in vitro* and some of these were dealt with in the previous section (Section 6.1.5). However, these factors were considered in relation to stem internodal grafts, and therefore it cannot be assumed that the conditions recognized as being conducive to graft formation with those tissues, would operate with thin cell layer grafts. Consequently, early experiments in this chapter were concerned with establishing the optimum conditions necessary to ensure thin cell layer graft formation. Results from Section 6.2.3 gave some indication as to the optimum explant position on the agar, with respect to graft development. It was found that insertion of the grafted explant into the agar, that is, vertical incubation compared to horizontal incubation, encouraged organogenesis. Similarly, reduction in the size of the donor tissue used, so that the grafted donor tissue covered approximately 60% of the recipient tissue, resulted in the formation of more shoots and roots (from the recipient tissue), than on explants where the donor tissue covered 100% of the recipient tissue (Section 6.2.4). In addition, this increase in organogenesis was accompanied by a decrease in graft formation with the species and cultivars investigated. Thus, organogenic expression would appear to be an undesirable event when graft formation is required. Garner (1979) reports on ex-

ceptional scion vigour as preceding incompatibility symptoms, and suggests that exceptional growth of either component of a graft may be a symptom of incompatibility. This is supported by Neilson-Jones (1969) who refers to disharmony between stock and scion being shown by the development of buds and suckers on the stock, which tend to overgrow and suppress the development of the scion. The question arises as to whether this is a cause or an effect response. The production of shoots and roots from either the stock or scion component of a graft could be the result of a graft that has failed or could be indicative of a graft that will fail, because hormones and other metabolites have been utilised in order to realize organogenic potential.

The importance of maintaining polarity in order to achieve graft development between two tissues was also indicated in Section 6.2.3, where graft establishment was zero when donor tissue was excised from stem tissue and replaced onto the appropriate recipient tissue (that is, the stem from which it was removed), but with the polarity reversed. It would seem that the polar differences within the tissues, due to their original orientation in the source plant are sufficient to impede, or prevent, any development towards graft formation.

The next factor which was considered was the need for growth regulators. As discussed in Section 6.1.5 *in vitro* autograft establishment (between stem internodal tissue) was favoured by the presence of auxin, and there were indications that apical application of auxin was preferable, the assumption being that an auxin flow across the graft union is thereby established promoting

the formation of vascular tissue. This formation of vascular tissue has been seen as a critical structural event in the formation of a successful graft (Yeoman et al., 1978). However, as previously discussed, thin cell layers present a different biological system: in contrast with stem internodal grafting, construction of the graft has not involved the disruption of an established vascular system. The presence of these severed vascular strands are considered important in that they are the preferred channels of transport of signals, for example auxin, which induce their own differentiation (Sachs, 1981). Observations have been made that cells not adjacent to severed vascular strands remain undifferentiated throughout graft development (Moore, 1984).

An indication of an auxin requirement was first observed in an early experiment (Section 6.2.7) when the donor tissue was seen to undergo no development and eventually go brown, when the medium was lacking in auxin. Further confirmation of this requirement was obtained when 2,4-D and BAP were the growth regulators used in the grafting medium (Section 6.2.9): graft formation being favoured by high levels of auxin ($2.0 mgl^{-1}$) in combination with either 0.2 or $2.0 mgl^{-1}$ cytokinin. This need for auxin was further expressed when 2,4-D was replaced with NAA, in that the majority of grafts established in this experiment (Section 6.2.10) had been cultured on media containing $2.0 mgl^{-1}$ NAA. It would seem, therefore that even though the thin cell layer grafting system differs from the system utilising sections of stem internodes, in both systems, successful graft formation is linked to the provision of an exogenous supply of auxin.

Histological analysis of successful thin cell layer heterografts revealed the presence of vascular tissue across the graft union. This observed vascular tissue was seen to take the form of meandering elongate procambial cells (Plate 6.2I, Section 6.2.16), xylem tracheary elements (Plate 6.2D, Section 6.2.16) and immature xylem elements (Plate 6.2H, Section 6.2.16). The presence of this vascular tissue would seem to infer that graft unions are strengthened through the differentiation of vascular tissue. The question arose as to whether the auxin supplied in the medium was responsible for inducing vascular differentiation through the establishment of auxin gradients within the explant.

In the culture of an intact stem internodal explant it could be assumed that any endogenous auxin present would flow within the vascular tissue towards the basipetal end of the explant, and absorbed exogenous auxin (from the medium) would similarly flow towards the basipetal end of the explant, possibly through both differentiated and undifferentiated tissue. Both NAA and 2,4-D move like IAA in a basipetally polar fashion but at a slower rate (Jacobs, 1979). Although this basipetal polar movement of auxin is exceedingly strong and stable, it can be affected by gravity (Section 6.1.5) and by the presence of other gradients within the tissue. The concept of morphogens diffusing along concentration gradients as suggested by Warren Wilson (1978) was outlined in Section 1.3.1. It is possible that exogenous auxin entering the recipient tissue of a thin cell layer heterograft would be faced with two distinct gradients. As auxin at a wounded surface can be destroyed by peroxidase acting as IAA-

oxidase (Iversen et al., 1970), it is likely that the auxin levels at the ends of the grafted explant would be low, thus providing one sink for the establishment of a concentration gradient. In addition, the auxin levels at the grafted surface are also likely to be low for the same reasons, thus providing an alternative sink and therefore, an alternative gradient. This gradient, together with the effect of gravity through horizontal incubation, could lessen the degree to which basipetal auxin movement occurs and result in the movement of auxin up through the tissue towards the graft union. This latter movement would probably be favoured by the fact that the exogenous auxin is NAA and not IAA, as IAA basipetal polar movement is least affected by other factors (Jacobs, 1979).

Vascular differentiation, in this investigation, was seen to occur initially at the ends of the grafted area (Plate 6.2C). There is evidence which suggests that applied physical pressure can promote cellular differentiation: callus systems derived from bark strips of Populus trichocarpa and Pinus strobus were subjected to pressure by wrapping the entire stem with rubber grafting bands and this physical confinement was sufficient to induce xylem and phloem differentiation, characteristic of the species (Brown et al., 1962). Observations of thin cell layer grafts often revealed that PTFE tape, used to secure the grafts, was stretched more tightly around the corners of the explant than in any other part of its circumference, thus subjecting the corners of the grafted area to greater pressure than the central area. In this way, auxin gradients and applied physical pressure could account for the initial appear-

ance of vascular tissue at the ends of the grafted area. Once vascular differentiation had occurred in these areas, this would induce other gradients within the undifferentiated tissue, along which further vascular differentiation could take part.

There are possibly further explanations as to this system's requirement for auxin, besides its suggested role in inducing vascular differentiation. Experiments with thin tissue slices of potato leaves have indicated that auxins may well overcome the reduced capabilities of thin tissue slices to perform cell divisions (Rosenstock et al., 1978). The implication is that low levels of endogenous hormones, in particular, auxin, hinder the tissue's ability to undergo wound healing. This reduced capability is likely to be further exacerbated by auxin destruction at the wound surface by peroxidase, and as there is evidence that peroxidase is present in relatively large concentrations in the epidermis of plants (Andreae et al., 1960; Zenk et al., 1964), this could further explain the need to provide exogenous auxin in a thin cell layer grafting system.

As reported in Section 6.2.11 (Table 6.10), increasing the concentration of cytokinin (BAP) in the culture medium resulted in increased graft formation under two of the light and temperature regimes tested, and maximum graft establishment was found to occur when explants were cultured on a medium containing $2.0 mgl^{-1}$ BAP (equal in concentration to the auxin present). With the other two remaining light and temperature regimes, there was little difference in the number of successful grafts obtained with differing con-

centrations of cytokinin. There is disagreement in the literature as to the role of cytokinins in vascular differentiation, and hence their possible role in graft formation. Parkinson et al., (1982) found that the presence of kinetin in the culture medium stimulated graft development through increasing the amount of vascular tissue in cultured internodes of Lycopersicon, Datura and Nicandra, but only in the presence of auxin. Similarly, Earle (1968) reported some stimulation of vascular regeneration when kinetin was added to the medium on which small pith explants of Coleus blumei were cultured. In contrast, Fosket et al., (1964) found that kinetin inhibited vascular differentiation in pith explants of Coleus blumei at all concentrations tested, and suggested that the observed inhibition could be interpreted as resulting from an inhibition of polar auxin transport. They stressed the importance of distinguishing between the effect of a substance on cambial activity, and the effect of the same substance on xylem differentiation and suggest that this might account for the conflicting reports in the literature. The results obtained in this investigation, show that the presence of BAP in the culture medium, in combination with auxin, either improved, or at the least did not negate, the stimulatory effect of auxin on graft formation. However, there was no direct evidence that the improvement obtained in graft establishment was due to increased vascular regeneration; whatever the effect of BAP, it was achieved in combination with auxin, and not in isolation.

Inclusion of GA_3 in the grafting medium resulted in reduced graft formation (Table 6.14, Section 6.2.14). These results agree with those of Parkinson et al., (1982) who found that the reduction in graft formation was due to GA_3-mediated inhibition

of vascular differentiation in internodal grafts of Lycopersicon, Datura and Nicandra in vitro. As with cytokinins, there is disagreement in the literature as to the role of GA_3 in vascular differentiation. For example, Roberts et al., (1965) found that culture of internodal tissue slices from Coleus blumei (Benth) stems on medium containing GA_3 resulted in increased formation of wound vessel members. It is suggested that the xylogenic effect of exogenous GA_3 may be due to either increased diffusible auxin (Kuraishi et al., 1964), decreased IAA oxidase (Pilet, 1957), or decreased peroxidase activity (McCurie et al., 1959). As this study was more concerned with the formation of grafts and their subsequent regeneration, as a means of producing chimeras, extensive histological analysis was not carried out on all grafts, therefore there is no direct evidence supporting the relationship between increased GA_3 levels and decreased vascular differentiation as the explanation for reduced graft formation. However, as shown in Table 6.14 (Section 6.2.14), grafting success was markedly reduced when GA_3 was present in the medium.

Consideration of Table 6.10 (Section 6.2.11) shows that maximum graft establishment between diploid and tetraploid tissue took place at a temperature of 22 ± 1°C with a 16h daylength and a light intensity of 85$\mu Mm^{-2}S^{-1}$PAR (with two of the three media tested). A review of the limited literature on in vitro grafting reveals that the temperature generally used falls within the range of 22°C and 27°C (Navarro et al., 1975; Negueroles et al., 1979; Shu-Ching Huang et al., 1980; Parkinson et al., 1982). Light regimes used by these workers varied with only Navarro et al.,

(1975) investigating the effect of light intensity on graft formation in Citrus. More recently, Mampouya (1983) confirmed that light was an important factor in achieving successful micrografts of Citrus species. It is probable that light and temperature are factors which are very dependent on other cultural conditions, and can only be looked at in interaction with other conditions, and not in isolation.

The stages involved in graft development were discussed in Section 1.3.1, however, these are stages as seen occurring during the development of a graft union between stem internodal components, and it does not necessarily follow that the stages of development of a union in a thin cell layer system would be the same. It is well recognized that the process of graft development generally begins with the formation of a necrotic layer, which in the majority of successful grafts disappears, as the graft progresses (McCully, 1983). In this present investigation, histological analysis of thin cell layer heterografts revealed in a number of the grafts a darker staining layer, which tended to be located on the donor tissue side of the graft interface (Plate 6.2I, Section 6.2.16). This was not positively identified as a necrotic layer, in that its composition was not determined, however, many of the later sections revealed this layer being penetrated by cell proliferation and differentiation, and consequently disappearing. It has been argued that necrotic layer removal is essential for the attainment of a strong graft union, however grafts have been successful where the necrotic layer has persisted (Section 1.3.1). Plate 6.2G (Section 6.2.16) shows a section through a thin cell layer heterograft where the darker staining

layer has remained throughout the development of the graft. It would appear that this explant was organogenically competent, although this ability seemed to be an expression of the donor tissue alone. Closer observation of this section (Plate 6.2I, Section 6.2.16) revealed vascular differentiation occurring within the donor tissue but ceasing within five to six cells of the recipient tissue.

It has been suggested that the stimulatory effect of a developing shoot on the regeneration of vascular tissue is due to the production of morphogens, especially auxins and gibberellins (Wetmore et al., 1955). Alternatively, Walker (1983) proposes a system of developmental control whereby cells mutually influence one another directly by a local exchange of positional information. Such information may be target specific morphogens rather than concentration gradients. Similarly, Moore et al., (1981) suggests that direct cellular communication could be a requisite for vascular development and that a persistent necrotic layer could prevent that communication, and therefore vascular differentiation. Plate 6.2G (Section 6.2.16) suggests that whatever was stimulating vascular differentiation in the donor tissue, failed to elicit a similar response in the recipient tissue. It is possible that if the darker staining layer is assumed to be a barrier layer, its presence insulated the graft partners and prevented the direct cellular communication that perhaps is essential for vascular differentiation. On the other hand, bearing in mind Walker's proposal (1983), there is the possibility that morphogens generated from diploid tissue and capable of inducing a developmental

response in diploid tissue, are not capable of bringing about a similar response in tetraploid tissue. Thus Plate 6.2G (Section 6.2.16) is representative of a grafted explant where two different tissues have adhered together, and one tissue is undergoing organogenesis independently of the other. Although a strong graft union existed, the grafted explant would seem to be composed of two units functioning separately rather than one unit functioning together, at least as far as regeneration is concerned. Moore et al., (1981a, 1981b) have shown that cut surfaces are capable of adhesion to non-living materials, and suggest the basis for graft cohesion, in the initial stages, is due to the deposition of cell wall material. It is possible that the grafted explant represented by Plate 6.2G (Section 6.2.16) provides an example of a graft where strength of union results from deposition of cell wall material. Development of the graft has occurred, even with the presence of a barrier layer, but failure of the graft may occur later, due to the inability of the two graft components to participate in direct cellular interaction.

The second stage in the process of graft development is generally recognized as callus proliferation (McCully, 1983), and as discussed in Section 1.3.1, its importance in the process of securing successful grafts has been argued. The majority of thin cell layer heterografts examined in this present investigation, revealed the presence of a callus bridge separating the donor tissue from the recipient tissue. As a general rule, it would appear that cells contributing to this callus bridge were derived from both components of the graft (Section 6.2.16) and initiation

of callus formation originated at the ends of the tissues of the grafted explants (Plate 6.2A, Section 6.2.16).

As discussed in Section 1.3.1, stem epidermal cells have been shown to be incompetent to dedifferentiate and form a graft (Walker et al., 1985). The authors' work involved the binding together of stem internodes so that the outer epidermal cells were forced into contact. Their results showed that such intact epidermal surfaces were incapable of dedifferentiation. Dedifferentiation and subsequent graft formation only took place when the epidermis from the surface of both internodes was removed. No attempt was made in this investigation to graft outer epidermal surfaces, however the relatively successful formation of thin cell layer grafts does show the competence of sub-epidermal cells to undergo cellular responses which can eventually lead to graft formation.

Results from experiments described in Section 6.2.13 indicate that the diploid species respond more favourably to grafting than the tetraploid species. General observations during the course of these grafting experiments revealed that browning of the epidermal layer (the donor tissue), was frequently noted when an autograft between two tetraploid components was constructed. These observations resemble those described in Section 5.5 where necrosis to varying degrees was observed within grafts of single and mixed callus. The results obtained in this investigation would tend to support the argument proposed in Section 5.5, that the tetraploid species is more sensitive to wounding than the diploid

species, and, in this case, this sensitivity results in impeding graft formation.

As reported in Section 6.2.15 an age effect on grafting was observed in that the use of younger tissue was seen to result in a greater number of successful grafts being formed in a shorter time. Mampouya (1983) and Jonard (1986) report on the influence of age of tissue on graft establishment. Rosenstock et al., (1978) suggested that the ability of a tissue to respond to wounding with induced mitosis is subject to an aging process, which begins at an early stage in the development of an organ. The authors propose that the aging of the tissue affects the source of phytohormones available in the unreactive tissue beneath the reactive cells in the wounded tissues. Aloni et al., (1985) in their study on wound-induced xylem regeneration in Zea mays L. found that parenchyma cells lose their ability to redifferentiate at a very early stage in their development, and that only very young stem tissues responded to wounding and formed fully regenerated wound vessel elements around the wound. Thus it is possible that the supply of endogenous phytohormones in the six week old tissue was sufficiently depleted to affect that tissue's response to wounding, and this in turn would affect the eventual graft formation.

The pattern of development most commonly observed in the formation of thin cell layer heterografts would seem to be similar to that described by other workers for other tissues (Jeffree et al., 1983; McCully, 1983; Moore, 1983). In this study of thin cell layer heterografts, there appears to be the production of some form of barrier layer (necrotic layer). Removal of this layer seemingly

depends on cell division for it is penetrated and eventually disappears when callus formation and vascular differentiation occur. Furthermore, in most grafts analysed, the formation of a callus bridge took place with the eventual development of vascular tissue from the cells within that callus bridge. However, there was evidence to suggest that the donor and recipient components of the graft can form a strong graft union independent of these events, although such a process of cohesion may eventually fail due to lack of vascular connections, and presumably direct cellular communication. For example, Vanilla orchid grafts survived until put under transpirational stress, the parenchyma cells at the union apparently providing enough flow in normal circumstances (Musik, 1958).

CHAPTER 7

REGENERATION STUDIES

7.1. INTRODUCTION

A necessary step in the *in vitro* synthesis of chimeral plants is the achievement of regeneration from a chimeral system. Maximum recovery of chimeral shoots from a chimeral system can only occur if that system has been provided with conditions necessary to attain maximum regeneration potential. Thus, it was important to ascertain the cultural conditions required to maximise regeneration from both the diploid and tetraploid species of potato which were to be combined in *in vitro* chimeral associations.

7.2. REGENERATION OF INTERNODAL STEM SEGMENTS

This experiment was concerned with evaluating the cultural conditions necessary to achieve maximum regeneration from stem internodal explants.

Stems of actively growing plantlets derived from shoot-tips which had been in culture for three weeks were used to provide internodal segments of approximately 8mm in length. These segments were inoculated onto agar so that the entire length of the tissue was in contact with the medium. The basic culture procedure was as outlined in Section 2.4.

A wide range of media was used; these were selected on the basis of previous successes in achieving regeneration from potato tissue (Wang *et al.*, 1975; Lam, 1975; O'Hara, pers. comm.). In all cases, basal culture media (Section 2.2) supplemented with 2% sucrose (w/v) were used with additions as shown in Table 7.1.

TABLE 7.1

REGENERATION MEDIA	GROWTH REGULATORS ($MG\ L^{-1}$)	OTHER ADDITIONS (GL^{-1})
E31	0.2 2,4-D + 2.0 zeatin	1.0 casein hydrolysate
E41	As E31 but less 2,4-D	As E31
E32	0.2 2,4-D + 2.0 zeatin riboside	1.0 casein hydrolysate
E42	As E32 but less 2,4-D	As E32
4ZR	4.0 zeatin riboside	-
1K	1.0 kinetin	-
10K	10.0 kinetin	-
.4B	0.4 BAP	-

E31/E41 and E32/E42 were both two-stage procedures in which explants were cultured on stage-one media (E31 or E32) for five to seven days before transfer to stage-two media (E41 or E42). Three light regimes were used: $5\mu Mm^{-2}s^{-1}$PAR and a 8h daylength; 20 and $85\mu Mm^{-2}s^{-1}$PAR, both 16h daylength, and all cultures were incubated at 22 ± 1°C. The species and cultivars used in this investigation were S. sparsipilum, S. chacoense, S. tuberosum ssp. tuberosum cv. Fortyfold, cv. Pentland Ivory, cv. Pentland Squire and cv. Majestic. The number of explants in any one treatment was 16. Results were assessed at regular intervals throughout the period of culture and final assessment was made after 90 days of culture.

Within 14 days of culture, the majority of explants had expanded and had developed swollen ends. Callus was beginning to form

on these ends. At this stage the morphogenic response was limited, mainly occurring when explants were cultured under $85\mu Mm^{-2}s^{-1}$PAR light intensity and on media containing zeatin riboside. After 28 days of culture most explants had produced callus at the cut ends. Explants cultured on media containing cytokinin only, produced very little callus, especially where the cytokinin content was $10mgl^{-1}$ kinetin. There was some increase in callus production as light intensity increased, with the E32/E42 procedure seemingly favouring callus formation. Shoot and root production had increased since day 14, the maximum response occurring on media containing zeatin riboside.

After 40 days of culture, there had been little or no organogenic response by the diploid species subjected to the E31/E41 and E32/E42 procedures. Tetraploid species, however, had produced roots and shoots when cultured on these media, though a superior organogenic response was expressed after culture on media containing $4mgl^{-1}$ zeatin riboside (4ZR). High levels of kinetin ($10mgl^{-1}$) had an inhibitory effect on regeneration in tetraploid species compared with the diploid species. As light intensity increased from $5\mu Mm^{-2}s^{-1}$PAR to $85\mu Mm^{-2}s^{-1}$PAR, both shoot and root regeneration increased.

Final assessment after 90 days of culture revealed 4ZR as the superior medium for inducing maximum shoot and root regeneration in all species and cultivars under all conditions, with two exceptions (see Table 7.2). The exceptions were firstly S. tuberosum ssp. tuberosum cv. Majestic under $85\mu Mm^{-2}s^{-1}$PAR where

a lower concentration of zeatin riboside and/or the presence of 2,4-D in the media (E32/E42) encouraged 100% shoot production whereas culture on 4ZR medium resulted in 94% shoot production, and secondly, S. tuberosum ssp. tuberosum cv. Pentland Squire, under 5$\mu Mm^{-2}s^{-1}$PAR, expressed maximum shoot response (87%) with the E32/E42 procedure, compared with 69% on 4ZR. This final assessment confirmed and emphasised the differing regeneration responses expressed by the diploid and tetraploid species to the E31/E42 and E32/E42 procedures (see Plates 7.1A to 7.1F). S. tuberosum ssp. tuberosum cv. Fortyfold, unlike other tetraploids, showed little or no response to E31/E41 and E32/E42, and so in terms of regeneration behaved more like a diploid species in this instance. In general, multiple shoots were produced from the explants and the regeneration sites were located at the ends of the internodal segments.

TABLE 7.2. Influence of regeneration media on morphogenic response of internodal segments excised from the stems of different species, and cultivars of potato

SPECIES/ CULTIVAR	MEDIA	LIGHT & TEMP CONDITIONS	% EXPL FORMING SHOOTS	% EXPL FORMING ROOTS
S. sparsipilum	E31/E41	LL/LT(8)	0	0
S. sparsipilum	E32/E42	LL/LT(8)	0	0
S. sparsipilum	4ZR	LL/LT(8)	87	0
S. sparsipilum	1K	LL/LT(8)	12	12
S. sparsipilum	10K	LL/LT(8)	12	6
S. sparsipilum	.4B	LL/LT(8)	6	0
S. chacoense	E31/E41	LL/LT(8)	0	0
S. chacoense	E32/E42	LL/LT(8)	0	6
S. chacoense	4ZR	LL/LT(8)	50	19
S. chacoense	1K	LL/LT(8)	12	12
S. chacoense	10K	LL/LT(8)	12	6
S. chacoense	.4B	LL/LT(8)	31	0
S. tuberosum	E31/E41	LL/LT(8)	50	0
ssp. tuberosum	E32/E42	LL/LT(8)	69	12
cv. Majestic	4ZR	LL/LT(8)	87	0
cv. Majestic	1K	LL/LT(8)	19	19
cv. Majestic	10K	LL/LT(8)	6	0
cv. Majestic	.4B	LL/LT(8)	12	12

TABLE 7.2. CONTINUED

SPECIES/ CULTIVAR	MEDIA	LIGHT & TEMP. CONDITIONS	% EXPL FORMING SHOOTS	% EXPL FORMING ROOTS
S. tuberosum	E31/E41	LL/LT(8)	75	0
ssp. tuberosum	E32/E42	LL/LT(8)	81	0
cv. Pentland squire	4ZR	LL/LT(8)	69	0
cv. Pentland squire	1K	LL/LT(8)	50	0
cv. Pentland squire	10K	LL/LT(8)	0	0
cv. Pentland squire	.4B	LL/LT(8)	19	0
S. tuberosum ssp.	E31/E41	LL/LT(8)	81	0
tuberosum cv.	E32/E42	LL/LT(8)	75	0
Pentland Ivory	4ZR	LL/LT(8)	100	0
Pentland Ivory	1K	LL/LT(8)	19	6
Pentland Ivory	10K	LL/LT(8)	0	0
Pentland Ivory	.4B	LL/LT(8)	25	19
S. tuberosum	E31/E41	LL/LT(8)	0	0
ssp. tuberosum	E32/E42	LL/LT(8)	0	0
cv. Fortyfold	4ZR	LL/LT(8)	50	0
cv. Fortyfold	1K	LL/LT(8)	0	19
cv. Fortyfold	10K	LL/LT(8)	0	6
cv. Fortyfold	.4B	LL/LT(8)	0	0

TABLE 7.2. CONTINUED

SPECIES/ CULTIVAR	MEDIA	LIGHT & TEMP. CONDITIONS	% EXPL FORMING SHOOTS	% EXPL FORMING ROOTS
S. sparsipilum	E31/E41	LL/LT	0	0
S. sparsipilum	E32/E42	LL/LT	0	0
S. sparsipilum	4ZR	LL/LT	100	100
S. sparsipilum	1K	LL/LT	25	12
S. sparsipilum	10K	LL/LT	25	0
S. sparsipilum	.4B	LL/LT	0	0
S. chacoense	E31/E41	LL/LT	31	0
S. chacoense	E32/E42	LL/LT	0	0
S. chacoense	4ZR	LL/LT	81	81
S. chacoense	1K	LL/LT	50	37
S. chacoense	10K	LL/LT	12	0
S. chacoense	.4B	LL/LT	69	56
S. tuberosum	E31/E41	LL/LT	94	44
ssp. tuberosum	E32/E42	LL/LT	100	100
cv. Majestic	4ZR	LL/LT	100	100
cv. Majestic	1K	LL/LT	50	37
cv. Majestic	10K	LL/LT	12	0
cv. Majestic	.4B	LL/LT	31	19

TABLE 7.2. CONTINUED

SPECIES/ CULTIVAR	MEDIA	LIGHT & TEMP. CONDITIONS	% EXPL. FORMING SHOOTS	% EXPL. FORMING ROOTS
S. tuberosum	E31/E41	LL/LT	0	19
ssp. tuberosum	E32/E42	LL/LT	19	0
cv. Pentland Squire	4ZR	LL/LT	94	50
cv. Pentland Squire	1K	LL/LT	0	19
cv. Pentland Squire	10K	LL/LT	0	0
cv. Pentland Squire	.4B	LL/LT	0	19
S. tuberosum ssp.	E31/E41	LL/LT	87	100
tuberosum	E32//42	LL/LT	87	56
cv. Pentland Ivory	4ZR	LL/LT	100	100
cv. Pentland Ivory	1K	LL/LT	12	12
cv. Pentland Ivory	10K	LL/LT	6	0
cv. Pentland Ivory	.4B	LL/LT	25	25
S. tuberosum	E31/E41	LL/LT	0	19
ssp. tuberosum	E32/E42	LL/LT	19	0
cv. Fortyfold	4ZR	LL/LT	92	50
cv. Fortyfold	1K	LL/LT	0	19
cv. Fortyfold	10K	LL/LT	0	19
cv. Fortyfold	.4B	LL/LT	0	19

TABLE 7.2. CONTINUED

SPECIES/ CULTIVAR	MEDIA	LIGHT & TEMP. CONDITIONS	% EXPL. FORMING SHOOTS	% EXPL. FORMING ROOTS
S. sparsipilum	E31/E41	HL/LT	6	6
S. sparsipilum	E32/E42	HL/LT	7	0
S. sparsipilum	4ZR	HL/LT	100	100
S. sparsipilum	1K	HL/LT	19	6
S. sparsipilum	10K	HL/LT	44	19
S. sparsipilum	.4B	HL/LT	12	6
S. chacoense	E31/E41	HL/LT	31	6
S. chacoense	E32/E42	HL/LT	6	12
S. chacoense	4ZR	HL/LT	100	100
S. chacoense	1K	HL/LT	6	12
S. chacoense	10K	HL/LT	62	25
S. chacoense	.4B	HL/LT	75	81
S. tuberosum	E31/E41	HL/LT	94	69
ssp. tuberosum	E32/E42	HL/LT	100	81
cv. Majestic	4ZR	HL/LT	94	94
cv. Majestic	1K	HL/LT	25	12
cv. Majestic	10K	HL/LT	25	0
cv. Majestic	.4B	HL/LT	23	19

TABLE 7.2. CONTINUED

SPECIES/ CULTIVAR	MEDIA	LIGHT & TEMP. CONDITIONS	% EXPL. FORMING SHOOTS	% EXPL. FORMING ROOTS
S. tuberosum	E31/E41	HL/LT	87	6
ssp. tuberosum	E32/E42	HL/LT	100	69
cv. Pentland Squire	4ZR	HL/LT	100	100
cv. Pentland Squire	1K	HL/LT	44	25
cv. Pentland Squire	10K	HL/LT	19	0
cv. Pentland Squire	.4B	HL/LT	0	31
S. tuberosum	E31/E41	HL/LT	81	31
ssp. tuberosum	E32/E42	HL/LT	87	56
cv. Pentland Ivory	4ZR	HL/LT	100	100
cv. Pentland Ivory	1K	HL/LT	44	19
cv. Pentland Ivory	10K	HL/LT	19	0
cv. Pentland Ivory	.4B	HL/LT	31	6
S. tuberosum	E31/E41	HL/LT	0	0
ssp. tuberosum	E32/E42	HL/LT	25	19
cv. Fortyfold	4ZR	HL/LT	3	31
cv. Fortyfold	1K	HL/LT	0	12
cv. Fortyfold	10K	HL/LT	0	19
cv. Fortyfold	.4B	HL/LT	87	37

Key:

Media: MS + 2% sucrose + growth regulators as indicated in Table 7.1

Temperature: 22 ± 1°C

Light conditions: 8h daylength; 5μMm^{-2}s^{-1}PAR (LL/LT/8)

16h daylength; 20μMm^{-2}s^{-1}PAR (LL/LT)

16h daylength; 85μMm^{-2}s^{-1}PAR (HL/LT)

Number of replicates per treatment: 16

Period of culture: 90 days

Plate 7.1. Morphogenic responses of internodal stem segments isolated from several species and cultivars of potato after 60 days culture on a number of media containing zeatin and zeatin riboside in the absence and presence of 2,4-D

A. Shoot regeneration from S. tuberosum ssp. tuberosum cv. Pentland Squire (A) and cv. Majestic (B) after a two-stage culture procedure involving zeatin ($2.0 mgl^{-1}$) in the absence and presence of 2,4-D ($0.2 mgl^{-1}$) x 1

B. As Plate 7.1A but cultivars used are Pentland Ivory (A) and Fortyfold (B). cv. Fortyfold unlike other tetraploid species showed limited organogenic response to this culture procedure x 1

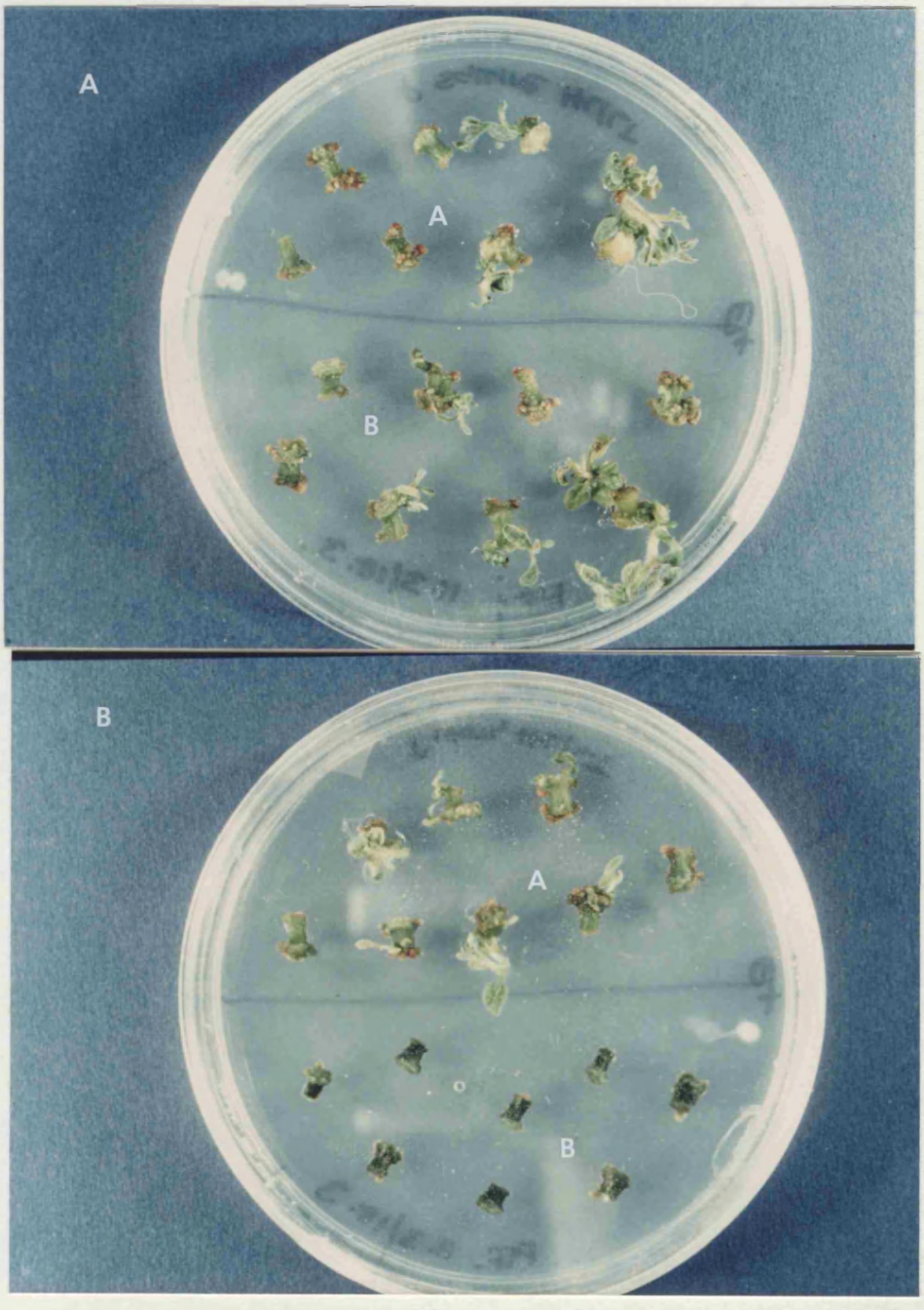

A
A
B
B
A
B

Plate 7.1. Morphogenic responses of internodal stem segments isolated from several species and cultivars of potato after 60 days culture on a number of media containing zeatin and zeatin riboside in the absence and presence of 2,4-D

C. Callus production from S. sparsipilum (A) and S. chacoense (B) after a two-stage procedure involving zeatin ($2.0 mgl^{-1}$) in the absence and presence of 2,4-D ($0.2 mgl^{-1}$). These explants showed no organogenic response to this culture procedure, merely developing callus, unlike the majority of explants of tetraploid origin shown in Plates 7.1A and 7.1B x 1

D. Shoot and root regeneration from S. tuberosum ssp. tuberosum cv. Pentland Squire (A) and cv. Majestic (B) after culture on a medium containing zeatin riboside ($4 mgl^{-1}$) x 1

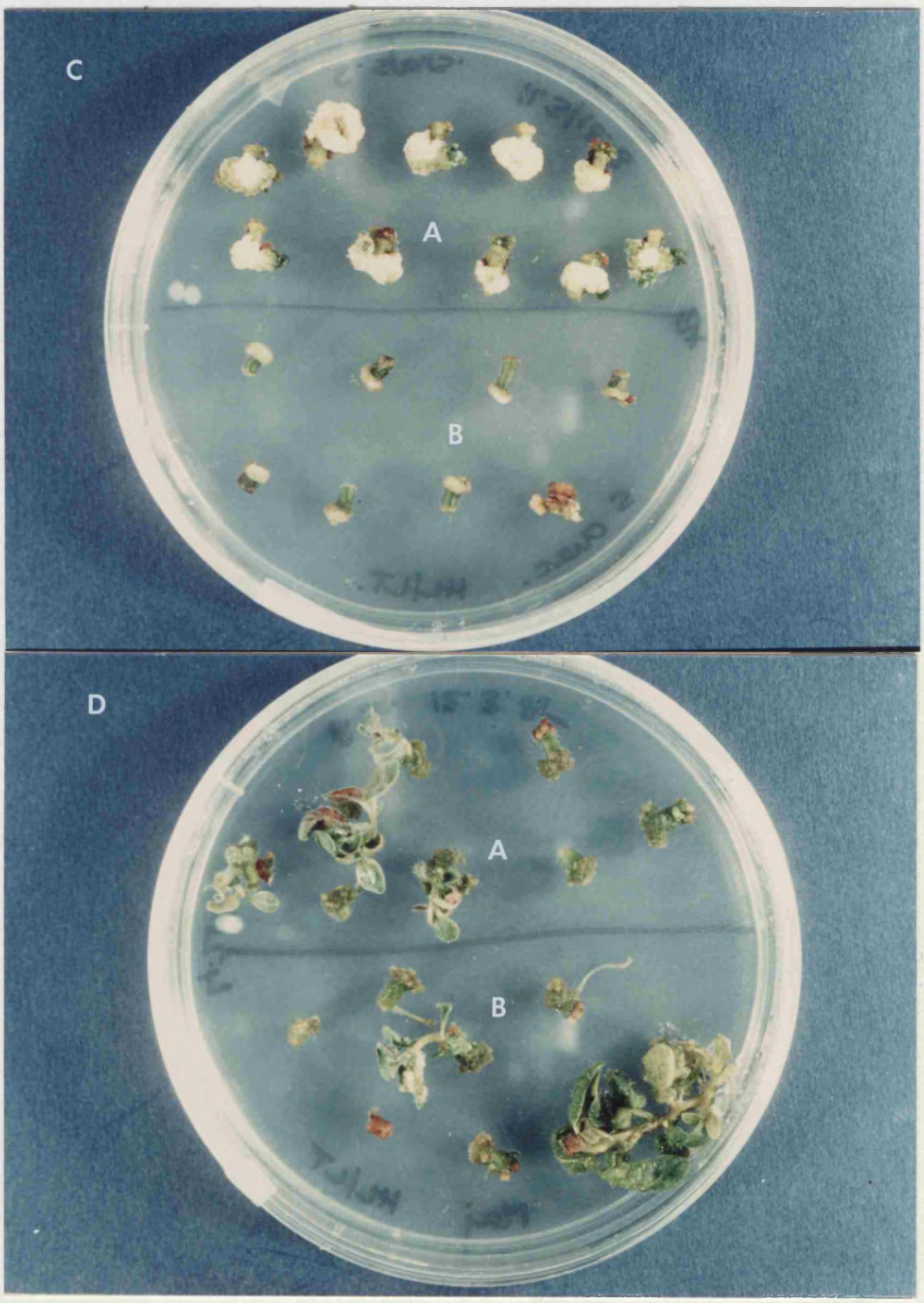
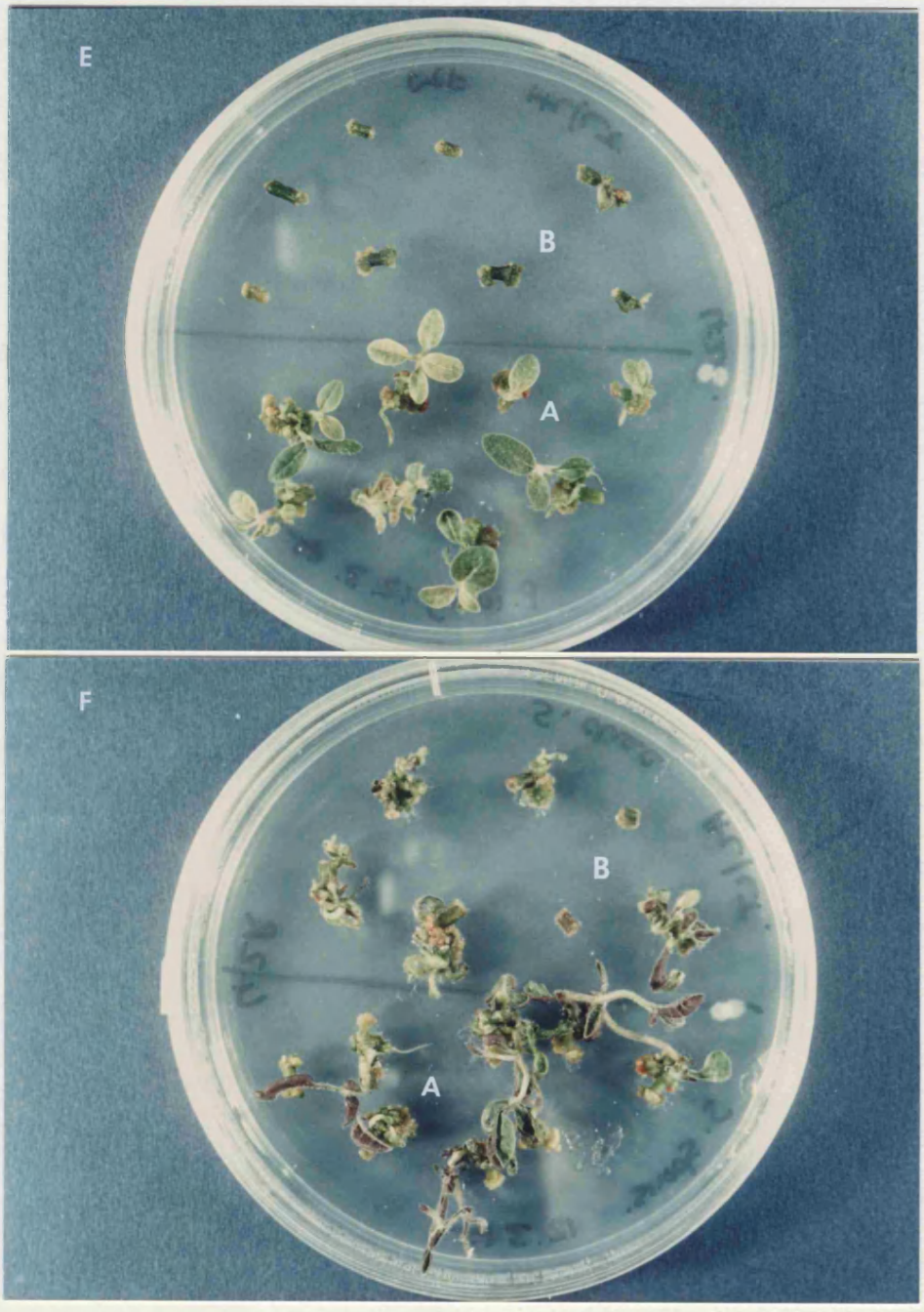

C
A
B
D
A
B

Plate 7.1. Morphogenic responses of internodal stem segments isolated from several species and cultivars of potato after 60 days culture on a number of media containing zeatin and zeatin riboside in the absence and presence of 2,4-D

E. Shoot and root regeneration from S. tuberosum ssp. tuberosum cv. Pentland Ivory (A) after culture on a medium containing zeatin riboside ($4mgl^{-1}$) cv. Fortyfold (B) unlike other tetraploid species showed limited organogenic response to this culture procedure x 1

F. As Plate 7.1D but species used are S. sparsipilum (A) and S. chacoense (B) x 1


E
B
A
F
B
A

7.3. EFFECT OF WOUNDING ON REGENERATION OF INTERNODAL STEM SECTIONS

Two experiments were carried out in an attempt to evaluate the influence wounding and the removal of a thin cell layer have on the regeneration of shoots and roots from internodal stem sections.

7.3.1. Shoot and root regeneration response after removal of a thin cell layer from internodal stem section

The aim of this investigation was to evaluate the effect that removal of a thin cell layer, that is, donor tissue, from an internodal stem segment, had on regeneration. It was necessary to establish whether this initial step in the grafting process influenced regeneration from the internodal stem segments.

Cultured explants were as described in Section 7.2. In addition, explants were also cultured where a section of the epidermal layer had been removed from the internodal stem segment (in general, this epidermal layer section measured 2mm x 8mm). These explants were inoculated onto agar so that the area from which the tissue had been removed, was uppermost. All other experimental details were as described in Section 7.2.

By day 14, most explants had developed swollen ends and callus was forming at the cut ends of the explants. After 28 days of culture, some shoot development had occurred, mainly from those explants cultured on media containing zeatin (E31/E41) or zeatin riboside (E32/E42 and 4ZR). Very little root development had occurred.

50 days of culture showed a further increase in shoot and root regeneration, with maximum shoot production being favoured by

the same media as described previously, that is E31/E41, E32/E42 and 4ZR. In this case, however, regeneration of shoots was not confined to the ends of the explants, in addition, shoots were regenerating from the boundary region where the wounded tissue joined with non-wounded tissue. Shoots also developed from the area where the thin cell layer had been removed but to a lesser extent. Regeneration from the ends of the explant involved multiple shoot formation, whereas elsewhere shoots developed singly or in small numbers. Results after 50 days indicated that the removal of a thin cell layer (epidermal layer) decreased the level of rooting. For example, under 16h daylength and light intensity of $85\mu Mm^{-2}s^{-1}$PAR (HL/LT) and, on 4ZR media, roots regenerated from cv. Pentland Ivory tissue decreased from 100% to 6%; similarly roots regenerated from S. chacoense decreased from 100% to 0% under the same conditions.

Results after 90 days of culture are shown in Table 7.3. On all media, where internodal stem segments had shown potential to regenerate roots, removal of a thin cell layer from such a stem segment inhibited that potential and led to reduced root development. This effect was more apparent with stem segments isolated from diploid species than with those from tetraploid species. The effect of thin cell layer removal on shoot regeneration from internodal stem segments was less uniform and varied with species, cultivar and media.

TABLE 7.3. Influence of epidermal layer on morphogenic response of internodal stem explants

SPECIES/ CULTIVAR	MEDIA	LIGHT + TEMP. CONDITIONS	% EXPLANTS FORMING SHOOTS		% EXPLANTS FORMING ROOTS	
			+ EPID. LAYER	- EPID. LAYER	+ EPID. LAYER	- EPID. LAYER
S. sparsipilum	E31/E41	LL/LT(8)	0	0	0	0
S. sparsipilum	E32/E42	LL/LT(8)	0	0	0	0
S. sparsipilum	4ZR	LL/LT(8)	87	100	0	0
S. sparsipilum	1K	LL/LT(8)	6	0	6	0
S. sparsipilum	10K	LL/LT(8)	6	0	6	0
S. sparsipilum	.4B	LL/LT(8)	0	50	0	0
S. chacoense	E31/E41	LL/LT(8)	0	0	0	0
S. chacoense	E32/E42	LL/LT(8)	0	0	6	0
S. chacoense	4ZR	LL/LT(8)	50	87	12	25
S. chacoense	1K	LL/LT(8)	12	0	12	0
S. chacoense	10K	LL/LT(8)	12	0	6	0
S. chacoense	.4B	LL/LT(8)	25	44	0	0
S. tuberosum	E31/E41	LL/LT(8)	50	75	0	25
ssp. tuberosum	E32/E42	LL/LT(8)	75	100	19	0
cv. Majestic	4ZR	LL/LT(8)	75	50	0	0
cv. Majestic	1K	LL/LT(8)	19	0	19	0
cv. Majestic	10K	LL/LT(8)	6	19	6	0
cv. Majestic	.4B	LL/LT(8)	12	6	19	0

TABLE 7.3. Influence of epidermal layer on morphogenic response of internodal stem explants

SPECIES CULTIVAR	MEDIA	LIGHT + TEMP. CONDITIONS	% EXPLANTS FORMING SHOOTS		% EXPLANTS FORMING ROOTS	
			+ EPID. LAYER	- EPID. LAYER	+ EPID. LAYER	- EPID. LAYER
S. tuberosum	E31/E41	LL/LT(8)	75	81	0	0
ssp. tuberosum	E32/E42	LL/LT(8)	75	100	0	19
cv. Pentland Ivory	4ZR	LL/LT(8)	100	100	0	0
cv. Pentland Ivory	1K	LL/LT(8)	19	33	6	0
cv. Pentland Ivory	10K	LL/LT(8)	0	17	0	0
cv. Pentland Ivory	.4B	LL/LT(8)	19	50	19	19
S. tuberosum	E31/E41	LL/LT(8)	81	87	0	0
ssp. tuberosum	E32/E42	LL/LT(8)	75	62	0	0
cv. Pentland Squire	4ZR	LL/LT(8)	69	50	0	0
S. tuberosum	1K	LL/LT(8)	50	0	0	0
ssp. tuberosum	10K	LL/LT(8)	0	6	0	0
cv. Pentland Squire	.4B	LL/LT(8)	19	19	0	6
S. tuberosum	E31/E41	LL/LT(8)	0	0	0	0
ssp. tuberosum	E32/E42	LL/LT(8)	0	0	0	0
cv. Fortyfold	4ZR	LL/LT(8)	50	19	0	0
cv. Fortyfold	1K	LL/LT(8)	0	0	25	0
cv. Fortyfold	10K	LL/LT(8)	0	0	6	0
cv. Fortyfold	.4B	LL/LT(8)	0	0	0	0

TABLE 7.3. CONTINUED

SPECIES/ CULTIVAR	MEDIA	LIGHT + TEMP. CONDITIONS	% EXPLANTS FORMING SHOOTS		% EXPLANTS FORMING ROOTS	
			+ EPID. LAYER	- EPID. LAYER	+ EPID. LAYER	- EPID. LAYER
S. chacoense	E31/E41	LL/LT	31	0	0	0
S. chacoense	E32/E42	LL/LT	0	0	0	0
S. chacoense	4ZR	LL/LT	87	100	75	0
S. chacoense	1K	LL/LT	50	6	37	6
S. chacoense	10K	LL/LT	12	50	0	25
S. chacoense	.4B	LL/LT	75	62	62	19
S. sparsipilum	E31/E41	LL/LT	0	6	0	0
S. sparsipilum	E32/E42	LL/LT	0	0	0	0
S. sparsipilum	4ZR	LL/LT	100	87	100	44
S. sparsipilum	1K	LL/LT	25	0	19	0
S. sparsipilum	10K	LL/LT	25	0	0	0
S. sparsipilum	.4B	LL/LT	0	0	0	0
S. tuberosum	E31/E41	LL/LT	94	100	44	81
ssp. tuberosum	E32/E42	LL/LT	100	100	100	100
cv. Majestic	4ZR	LL/LT	100	69	100	50
cv. Majestic	1K	LL/LT	50	6	31	6
cv. Majestic	10K	LL/LT	19	44	0	0
cv. Majestic	.4B	LL/LT	37	44	12	0

TABLE 7.3. CONTINUED

SPECIES/ CULTIVAR	MEDIA	LIGHT + TEMP. CONDITIONS	% EXPLANTS FORMING SHOOTS		% EXPLANTS FORMING ROOTS	
			+ EPID. LAYER	- EPID. LAYER	+ EPID. LAYER	- EPID. LAYER
S. tuberosum	E31/E41	LL/LT	87	81	100	69
ssp. tuberosum	E32/E42	LL/LT	94	81	62	44
cv. Pentland Ivory	4ZR	LL/LT	100	75	100	6
cv. Pentland Ivory	1K	LL/LT	6	31	12	25
cv. Pentland Ivory	10K	LL/LT	6	19	0	6
cv. Pentland Ivory	.4B	LL/LT	25	31	25	0
S. tuberosum	E31/E41	LL/LT	0	100	19	0
ssp. tuberosum	E32/E42	LL/LT	19	100	0	75
cv. Pentland Squire	4ZR	LL/LT	94	81	50	25
cv. Pentland Squire	1K	LL/LT	0	44	19	31
cv. Pentland Squire	10K	LL/LT	0	19	0	0
cv. Pentland Squire	.4B	LL/LT	0	19	25	6
S. tuberosum	E31/E41	LL/LT	0	19	19	0
ssp. tuberosum	E32/E42	LL/LT	25	0	0	0
cv. Fortyfold	4ZR	LL/LT	94	50	50	25
cv. Fortyfold	1K	LL/LT	0	0	19	0
cv. Fortyfold	10K	LL/LT	0	0	19	0
cv. Fortyfold	.4B	LL/LT	0	0	25	0

TABLE 7.3. CONTINUED

SPECIES/ CULTIVAR	MEDIA	LIGHT + TEMP. CONDITIONS	% EXPLANTS FORMING SHOOTS		% EXPLANTS FORMING ROOTS	
			+ EPID. LAYER	- EPID. LAYER	+ EPID. LAYER	- EPID. LAYER
S. chacoense	E31/E41	HL/LT	37	0	6	0
S. chacoense	E32/E42	HL/LT	6	25	12	0
S. chacoense	4ZR	HL/LT	100	100	100	0
S. chacoense	1K	HL/LT	6	0	12	0
S. chacoense	10K	HL/LT	69	31	25	6
S. chacoense	.4B	HL/LT	75	56	75	0
S. sparsipilum	E31/E41	HL/LT	6	0	0	0
S. sparsipilum	E32/E42	HL/LT	6	0	0	0
S. sparsipilum	4ZR	HL/LT	100	94	100	62
S. sparsipilum	1K	HL/LT	25	0	6	0
S. sparsipilum	10K	HL/LT	50	25	19	6
S. sparsipilum	.4B	HL/LT	12	0	6	0
S. tuberosum ssp. tuberosum cv. Majestic	E31/E41	HL/LT	100	0	62	0
	E32/E42	HL/LT	100	69	87	0
	4ZR	HL/LT	100	100	100	0
	1K	HL/LT	25	19	12	0
	10K	HL/LT	25	44	0	6
	.4B	HL/LT	25	44	19	0

TABLE 7.3. CONTINUED

SPECIES/ CULTIVAR	MEDIA	LIGHT + TEMP. CONDITIONS	% EXPLANTS FORMING SHOOTS		% EXPLANTS FORMING ROOTS	
			+ EPID. LAYER	- EPID. LAYER	+ EPID. LAYER	- EPID. LAYER
S. tuberosum	E31/E41	HL/LT	81	100	31	100
ssp. tuberosum	E32/E42	HL/LT	100	94	50	6
cv. Pentland Ivory	4ZR	HL/LT	100	100	100	6
cv. Pentland Ivory	1K	HL/LT	50	31	19	12
cv. Pentland Ivory	10K	HL/LT	19	62	0	12
cv. Pentland Ivory	.4B	HL/LT	31	44	6	12
S. tuberosum	E31/E41	HL/LT	87	87	6	0
ssp. tuberosum	E32/E42	HL/LT	100	44	75	0
cv. Pentland Squire	4ZR	HL/LT	100	81	100	0
cv. Pentland Squire	1K	HL/LT	44	50	25	25
cv. Pentland Squire	10K	HL/LT	12	19	0	0
cv. Pentland Squire	.4B	HL/LT	0	31	37	19
S. tuberosum	E31/E41	HL/LT	0	0	0	0
ssp. tuberosum	E32/E42	HL/LT	25	0	12	0
cv. Fortyfold	4ZR	HL/LT	25	50	25	19
cv. Fortyfold	1K	HL/LT	0	0	12	0
cv. Fortyfold	10K	HL/LT	0	0	19	0
cv. Fortyfold	.4B	HL/LT	75	0	25	0

Key:

Media: MS + 2% sucrose + growth regulators as indicated in Table 7.1

Temperature: 22 ± 1°C

Light conditions: 8h daylength; 5μMm^{-2}s^{-1}PAR (LL/LT(8))

16h daylength; 20μMm^{-2}s^{-1}PAR (LL/LT)

16h daylength; 85μMm^{-2}s^{-1}PAR (HL/LT)

Number of replicates per treatment: 16

Period of culture: 90 days

7.3.2. Adventitious shoot formation after removal of a thin cell layer from internodal stem section

The aim of this experiment was to examine more closely what effect removal of a thin cell layer had on adventitious shoot regeneration from an internodal stem segment.

Explants were treated in one of the following three ways:

(1) Cultured intact (as in Section 7.2)

(2) Cultured after thin cell layer removal (as in Section 7.3)

(3) As (2) but the thin cell layer is replaced onto the stem segment, with polarity maintained.

The experimental procedure was similar to that described in Section 7.3 except that explants were only cultured on 4ZR media (Section 7.2) under 85$\mu Mm^{-2}s^{-1}$PAR (16h daylength) at a temperature of 22 ± 1°C. S. tuberosum ssp. tuberosum cv. Pentland Ivory and S. sparsipilum were used in this investigation. For each of these three groups, 40 explants of each species were cultured. Explants were assessed after 14 and 50 days of culture.

After 14 days of culture the general response of the explants to culture was as described in Section 7.2 and 7.3.1. Shoot formation had occurred more readily from S. sparsipilum in all three experimental groups compared with S. tuberosum ssp. tuberosum cv. Pentland Ivory, and all shoots from S. sparsipilum had regenerated from the apical end of the explant, whereas the limited number of adventitious shoots regenerated from cv. Pentland Ivory had developed from the basal end of the explant.

The results after 50 days of culture are shown in Table 7.4. Results have indicated that shoot regeneration potential in S. sparsipilum is confined to the apical end of the explant and that neither removal of the epidermal layer nor removal and replacement of the epidermal layer affected shoot regeneration. However, with cv. Pentland Ivory, shoot regeneration potential was extended to both apical and basal ends of the intact internodal explant, but there was a greater production of shoots from the basal end (30% apical: 77% basal). Removal of the epidermal layer resulted in a slightly increased response at the apical end of the explant (50% apical: 71% basal). However, replacement of the epidermal layer resulted in reduced shoot regeneration at both the apical and basal ends, with the reduction at the apical end being more marked than that at the basal end of the explant (21% apical : 67% basal).

TABLE 7.4. Influence of epidermal layer on the extent and location of shoot regeneration from internodal stem explants

SPECIES AND CULTIVARS	TREATMENT 1: EXPLANT CULTURED INTACT			TREATMENT 2: THIN CELL LAYER REMOVED			TREATMENT 3: THIN CELL LAYER REMOVED AND THEN REPLACED		
	% EXPLANT WITH SHOOTS AT APICAL END	% EXPLANT WITH SHOOTS AT BASAL END	% EXPLANT WITH SHOOTS AT BOTH ENDS	% EXPLANT WITH SHOOTS AT APICAL END	% EXPLANT WITH SHOOTS AT BASAL END	% EXPLANT WITH SHOOTS AT BOTH ENDS	% EXPLANT WITH SHOOTS AT APICAL END	% EXPLANT WITH SHOOTS AT BASAL END	% EXPLANT WITH SHOOTS AT BOTH ENDS
S. tuberosum ssp. tuberosum cv. Pentland Ivory	30	77	7	50	72	22	22	67	5
S. sparsipilum	95	-	-	95	-	-	95	-	-

Key:

Media: MS + 2% sucrose (w/v) + zeatin riboside ($4mgl^{-1}$)

Temperature: $22 \pm 1^{o}C$

Light conditions: 16h daylength; $85\mu Mm^{-2}s^{-1}$ PAR

Number of replicates per treatment: 40

Period of culture: 50 days

7.4. HISTOLOGICAL EXAMINATION OF REGENERATION FROM INTERNODAL STEM SECTIONS

Internodal stem segments were cultured as described in Section 7.2. Those explants cultured on basal culture medium supplemented with 2% sucrose (w/v) and 4.0mgl^{-1} zeatin riboside under a light intensity of 85μMm^{-2}s^{-1}PAR (16h daylength) and temperature of 22 ± 1^{o}C, were taken out from culture at weekly intervals for histological procedures as described in Section 2.5, until shoot bud formation from the tissue was visible. S. tuberosum ssp. tuberosum cv. Pentland Ivory was selected for this examination. The purpose of this investigation was to determine the processes leading to adventitious shoot initiation for it was thought that such information would assist in clarifying the basis on which chimeral shoots might be formed.

Observations revealed active cell divisions in the vascular tissue of the stem, which had led to an increase in vascular tissue generally and the consequent formation of a continuous ring of vascular tissue, as opposed to discrete bundles. This, and the enlargement of the pith and cortical cells was observed after seven days of culture (Plate 7.2A). A further seven days of culture revealed extensive differentiation and areas of meristematic activity located between regions of differentiation and the outer layers of the tissue (Plate 7.2B). Subsequent sections confirmed the extent of this activity, that is, extensive differentiation within the cortical region of the explant and early indications of shoot organization near the explant surface (Plate 7.2C). Sections through other explants revealed similar

meristematic activity close to the explant surface, however, the actively differentiating cells, immediately beneath these areas of meristematic tissue were separated from the original region of vascular tissue by large, vacuolated cells (Plate 7.2D).

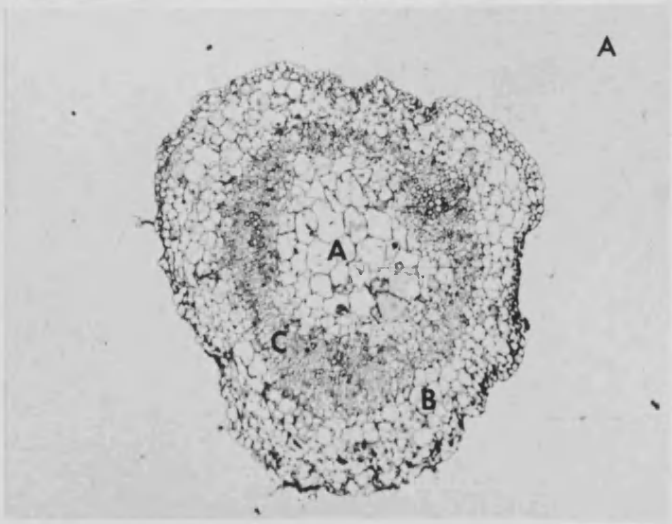
After 28 days of culture, developing shoot buds were observed around the perimeter of the explant (Plate 7.2E) with the cells undergoing extensive differentiation lying beneath them (Plate 7.2F). A close view of a shoot bud revealed the existence of a single tunica layer (Plate 7.2G).

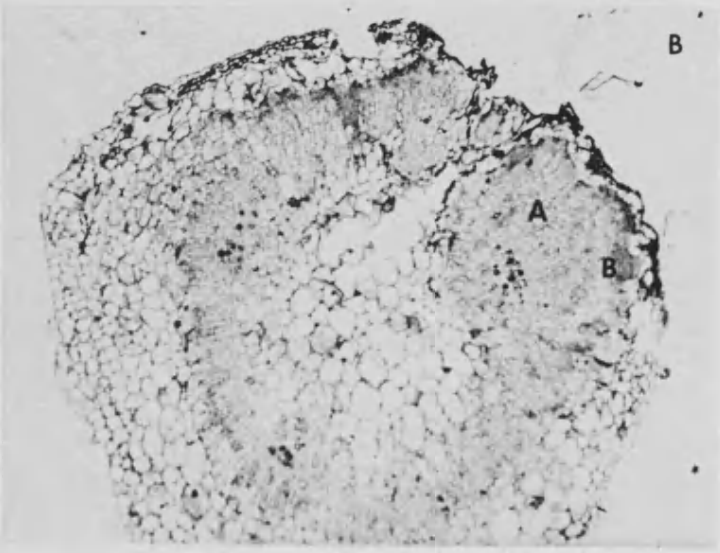
Plate 7.2. Histological examination of regeneration from internodal stem sections of S. tuberosum ssp. tuberosum cv. Pentland Ivory cultured on medium containing zeatin riboside ($4.0 mg l^{-1}$)

A. Development of the explant had resulted in the formation of a continuous ring of vascular tissue (C). This and the enlargement of the pith (A) and cortical cells (B) was observed after seven days of culture x 44

B. After 14 days of culture further differentiation had taken place (A) and areas of meristematic activity (B) were observed, often located between the regions of differentiation and the outer layers of the tissue x 109

C. Extensive differentiation in the cortical region of a stem explant (B) and early indications of shoot organization near the explant surface (A) x 109


A
A
C
B


B
A
B

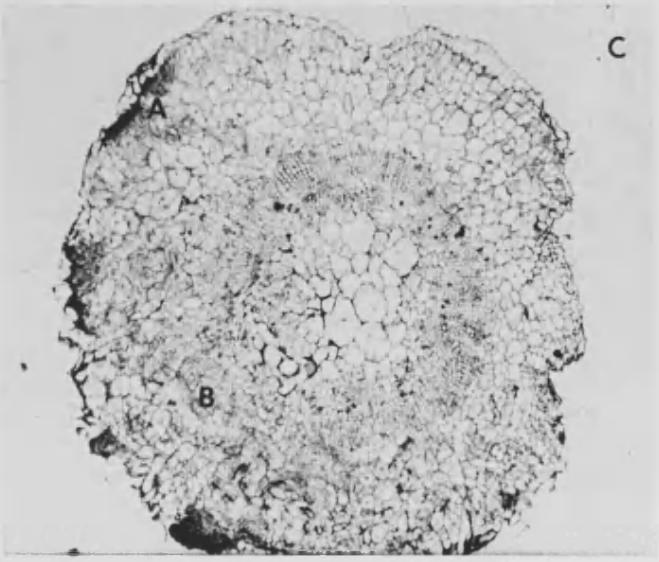

C
A
B

Plate 7.2. Histological examination of regeneration from internodal stem sections of S tuberosum ssp. tuberosum cv. Pentland Ivory cultured on medium containing zeatin riboside ($4.0 mgl^{-1}$)

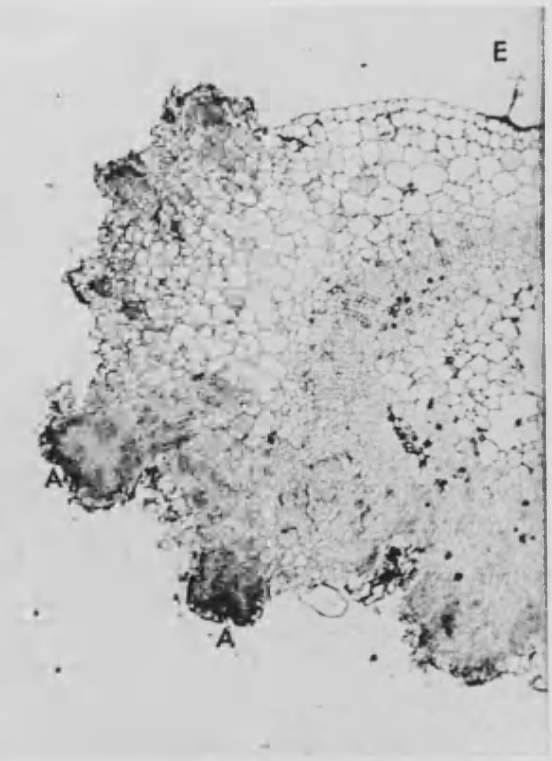
D. Areas of intense meristematic activity (A) separated from the original region of vascular tissue by large vacuolated cells (B) x 109

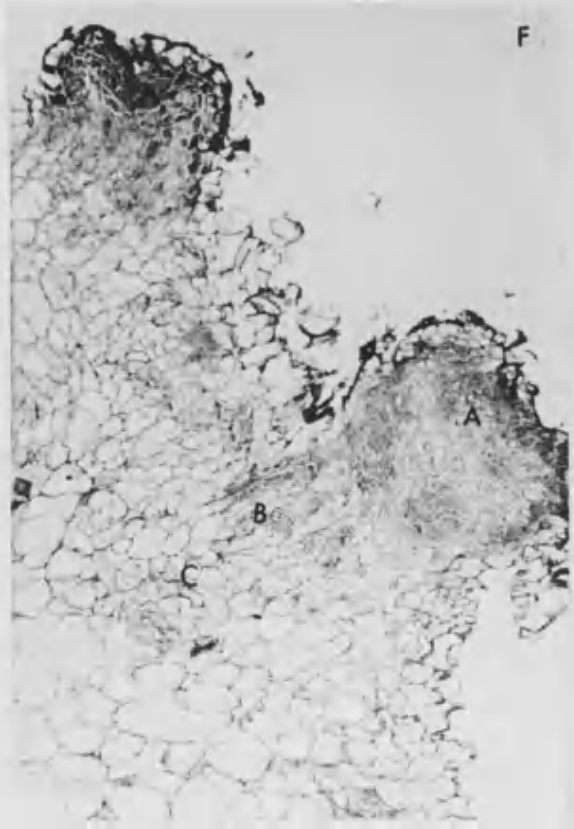
E. Developing shoot buds around the perimeter of the explant after 28 days of culture (A) x 44

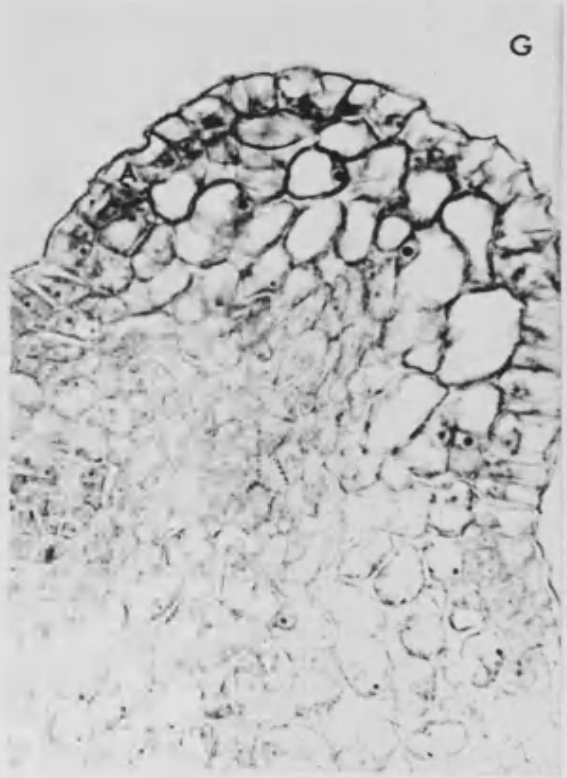
F. As E but showing the area beneath the developing shoot buds (A) where the cells are undergoing extensive differentiation revealing the formation of immature sieve tubes (B) and tracheary-type elements (C) x 109

G. Close view of a shoot bud, showing the formation of a single tunica layer x 437


D
A
B


E
A
A


F
A
B
C


G

7.5. MORPHOGENETIC POTENTIAL OF THIN CELL LAYERS

This investigation was carried out to assess the morphogenetic ability of thin cell layers isolated from stem sections of different species and cultivars of potato, as it was considered that this ability (or lack of it), would play a major contributory role in the formation of shoots from thin cell layer grafted explants.

Thin cell layers (three to six cells thick) were isolated from the internodal sections of stems dissected from plantlets derived from shoot-tip cultures, and inoculated onto agar with the outer surface uppermost. Culture media and conditions were as detailed in Section 7.2. The number of explants per treatment was 40. Assessment of results was made 14, 28, 50, 70 and 90 days after inoculation of explants onto agar.

After 14 days of culture, the majority of explants on the following media had shown some form of response: E31/E41; E32/E42; and 4ZR (Section 7.2). Early indications of possible organogenic potential were shown by small, green protuberances which were scattered over the surface of the explant. Between 14 and 28 days of culture, these protuberances could be seen as cellular proliferations over the entire epidermal surface. This was in contrast to other explants where expansion and some callus formation were the only response to culture (Plate 7.3A). Other thin cell layers had remained as they were at initiation of culture (Plate 7.3B), whereas some layers had produced callus and also a relatively large number of unicellular hairs (Plate 7.3C).

Further growth and expansion had occurred by day 50, seemingly favoured by the lower light intensities, and at this stage, S. chacoense and S. tuberosum ssp. tuberosum cv. Pentland Ivory were showing indications of shoot formation. These took the form of small green protuberances which had seemingly developed from the earlier cellular proliferations noted (Plate 7.3D). Between day 50 and day 70 shoot regeneration had occurred and as can be seen from Plate 7.3E, these shoots tended to be confined to the sides and ends of the expanded cell layers. Although multiple shoot formation was observed (Plate 7.3F), most shoots were regenerated singly or in groups of two or three. Roots were produced by a small proportion of those explants that had already produced shoot structures. With some explants division of the epidermal cells was restricted to a zone of the explant as illustrated by Plate 7.3G.

With the exception of S. chacoense and S. sparsipilum subjected to the two-stage E32/E42 and E31/E41 procedures respectively, shoot regeneration was confined to explants cultured under low light intensity (5 and 20$\mu Mm^{-2}s^{-1}$PAR). Table 7.5 shows only the explants and culture conditions where shoot regeneration was obtained. As Table 7.5 shows, the thin cell layers of the diploid species possessed greater morphogenetic potential than those of the tetraploid species, under the conditions tested.

Thin cell layers of S. chacoense cultured on E31/E41 under low intensity light ($5\mu Mm^{-2}s^{-1}$PAR) expressed greater organogenic response in terms of shoot regeneration than intact stem internodal explants (Section 7.2), under the same conditions. However, increasing

light intensity resulted in a decrease in shoot regeneration from the thin cell layers, but an increase in shoots regenerated from intact, stem internodal explants (Section 7.2). With the E32/E42 culture procedures, thin cell layers of S. chacoense regenerated shoots under all the levels of light intensity that were tested, whereas there was only minimal response (6%) under light intensity of $85\mu Mm^{-2}s^{-1}$PAR, when intact, stem internodal explants were cultured. Intact stem explants of S. sparsipilum showed very little response to the E31/E41 and E32/E42 culture procedures; this response was at its maximum at light intensity of $85\mu Mm^{-2}s^{-1}$PAR when 7% of the explants cultured produced shoots (Section 7.2). Conversely, thin cell layer explants of S. sparsipilum produced more shoots than intact stem explants when cultured with the E31/E41 and E32/E42 procedures; this response increased with increasing light intensity when the two-stage procedure was E31/E41, but decreased when the procedure was E32/E42. With all species and cultivars examined, culture on media containing $4mgl^{-1}$ zeatin riboside (4ZR) resulted in explants expressing little or no organogenic response.

TABLE 7.5. Adventitious shoot formation from thin cell layers excised from internodal stem segments of potato

SPECIES/ CULTIVARS	MEDIA	% EXPLANTS FORMING SHOOTS	LIGHT AND TEMPERATURE CONDITIONS
S. chacoense	E31/E41	42	LL/LT(8)
S. chacoense	E32/E42	45	LL/LT(8)
S. sparsipilum	E32/E42	11	LL/LT(8)
S. tuberosum ssp *tuberosum* cv. Pentland Ivory	E32/E42	8	LL/LT(8)
S. chacoense	E31/E41	8	LL/LT
S. chacoense	E32/E42	20	LL/LT
S. sparsipilum	E31/E41	8	LL/LT
S. sparsipilum	E32/E42	8	LL/LT
S. sparsipilum	4ZR	11	LL/LT
S. tuberosum ssp. *tuberosum* cv. Majestic	E32/E42	11	LL/LT
S. chacoense	E32/E42	33	HL/LT
S. sparsipilum	E31/E41	20	HL/LT

Key:

Media: MS + 2% sucrose (w/v) + different combinations of various growth regulators (see Table 7.1, Section 7.2)

Temperature: 22 ± 1°C

Light conditions: 8h daylength; 5μMm^{-2}s^{-1}PAR (LL/LT(8))

16h daylength; 20μMm^{-2}s^{-1}PAR (LL/LT)

16h daylength; 85μMm^{-2}s^{-1}PAR (HL/LT)

Period of culture: 90 days

Number of replicates per treatment: 40

Plate 7.3. Morphogenic response of thin cell layers excised from internodal stem segments of potato species after culture on a number of media containing zeatin and zeatin riboside in the absence and presence of 2,4-D

A. Thin cell layer of S. tuberosum ssp. tuberosum cv. Desiree after 33 days culture using a two-stage procedure involving zeatin ($2mgl^{-1}$) in the absence and presence of 2,4-D ($0.2mgl^{-1}$). This type of development did not result in any organogenic response x 25

B. Thin cell layer of S. tuberosum ssp. tuberosum cv. Fortyfold after 41 days culture on a medium containing zeatin riboside ($4mgl^{-1}$). There had been no change in the explant since the initiation of culture x 16

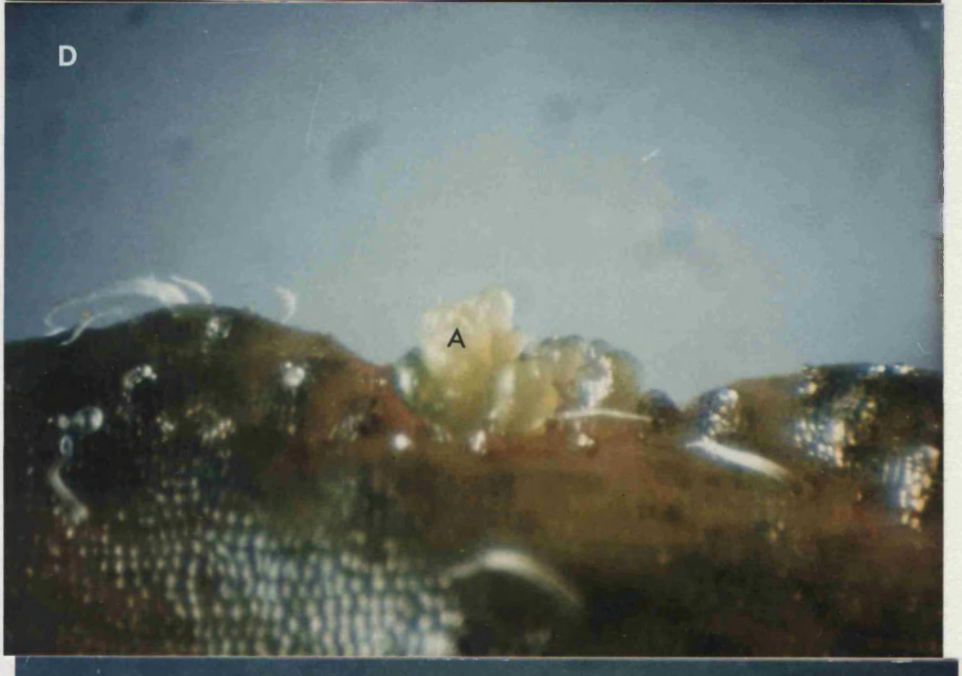
Plate 7.3. Morphogenic response of thin cell layers excised from internodal stem segments of potato species after culture on a number of media containing zeatin and zeatin riboside in the absence and presence of 2,4-D

C. Thin cell layer of S. tuberosum ssp. tuberosum cv. Majestic after 41 days culture using a two-stage procedure involving zeatin riboside ($2mgl^{-1}$) in the absence and presence of 2,4-D ($0.2mgl^{-1}$). The response shown by this explant was the production of callus and a large number of hairs x 16

D. Thin cell layer of S. chacoense after 50 days culture using a two-stage procedure involving zeatin ($2mgl^{-1}$) in the absence and presence of 2,4-D ($0.2mgl^{-1}$) showing small, green protuberances (A) which were indicative of shoot formation x 25

E. Thin cell layer of S. tuberosum ssp. tuberosum cv. Pentland Ivory after 70 days culture using a two-stage procedure involving zeatin riboside ($2mgl^{-1}$) in the absence and presence of 2,4-D ($0.2mgl^{-1}$). A single shoot had regenerated from the end of the explant; the callus phase was minimal x 6


C

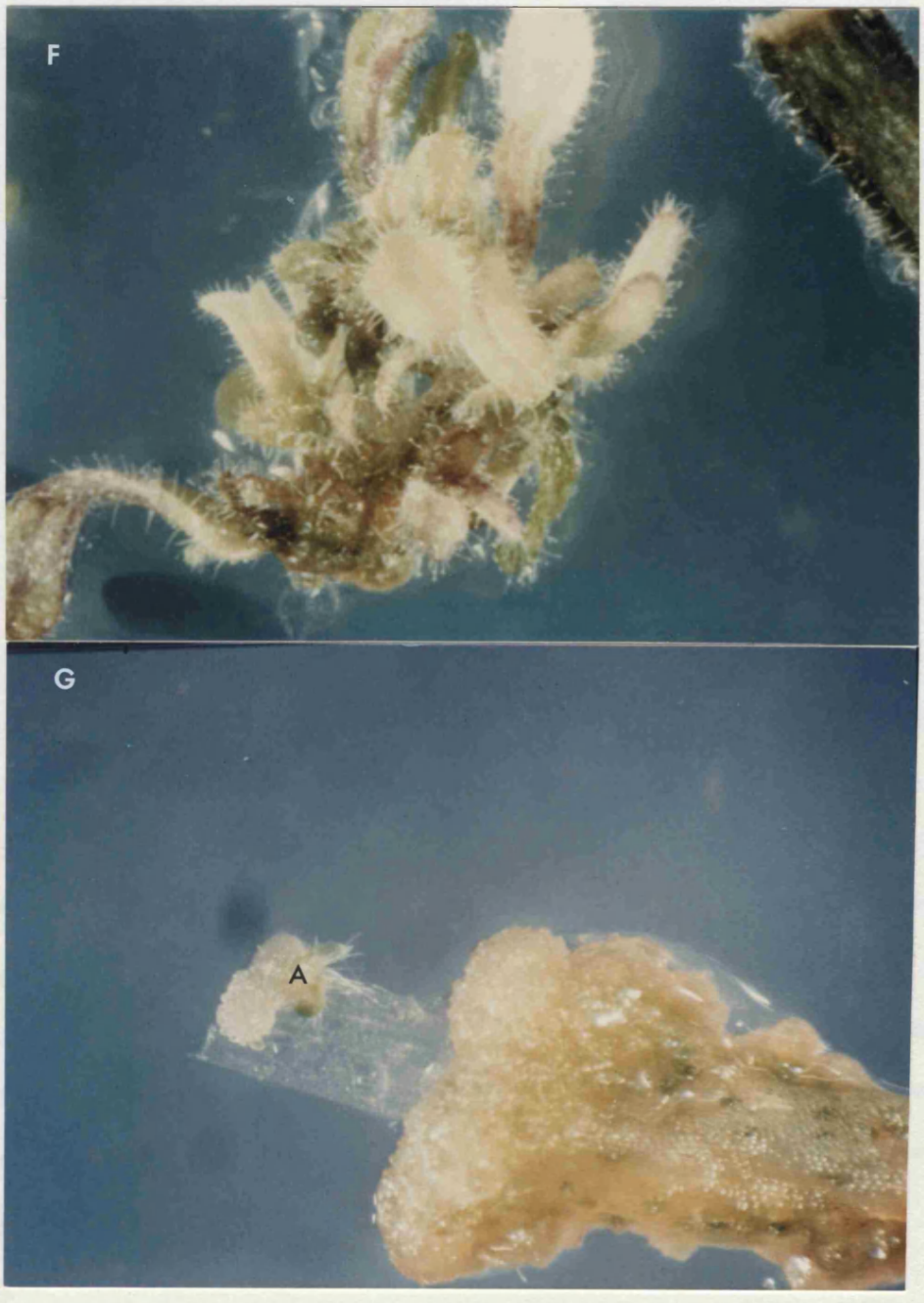

D
A


E

Plate 7.3. Morphogenic response of thin cell layers excised from internodal stem segments of potato species after culture on a number of media containing zeatin and zeatin riboside in the absence and presence of 2,4-D

F. Thin cell layer of S. sparsipilum after 70 days culture using a two-stage procedure involving zeatin riboside ($2mgl^{-1}$) in the absence and presence of 2,4-D ($0.2mgl^{-1}$) showing multiple shoot formation x 16

G. As Plate 7.3F except that single shoot regeneration (A) was restricted to a small, isolated region of the explant x 16


F
G
A

7.6. HISTOLOGICAL EXAMINATION OF REGENERATION FROM THIN CELL LAYERS

Thin cell layers were cultured as described in Section 7.5. Only those species and cultivars which had showed a positive organogenic response to culture (Section 7.5) were used in this investigation. Similarly, choice of media was based on results from Section 7.5 (Table 7.5). Explants were taken out from culture at regular intervals for histological procedures as described in Section 2.5.

It was hoped that this examination, in demonstrating which cells undergo division and participate in regeneration, would clarify the role of the epidermal layer in regeneration of adventitious shoots, and thus show that in thin cell layer grafts (epidermal grafts), it would not interfere with the formation of chimeral shoots.

On the fourth day of culture periclinal divisions were observed in the sub-epidermal layers and in places, layers of flattened cells were formed.(Plate 7.4A). After seven days in culture, the cytological processes which had already been observed were accentuated, anticlinal, periclinal and oblique divisions had occurred, thus producing zones of intense activity (Plate 7.4B). Although divisions in the uppermost epidermal layer (L1) are considered to be predominantly anticlinal, there were indications of periclinal divisions, however, such observations were restricted to a very small number of explants (Plate 7.4C). After ten days of culture, histological analysis revealed that the average thin cell layer explant had expanded from approximately three to six cell layers deep to approximately 15 to 20 cell layers deep (Plate 7.4D). A further four days of culture revealed that cell activity had resulted in the formation of groups

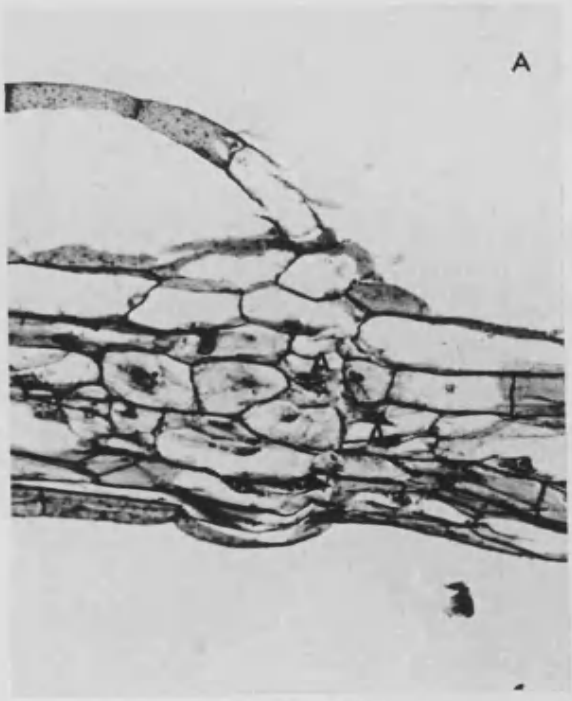
of small meristematic cells which formed domes at the explant surface (Plate 7.4E). A less direct form of development was observed in other explants where histological examination revealed the formation of nodules, within which cells were actively dividing and differentiating (Plate 7.4F). Zones of meristematic activity were observed within these nodules just beneath the surface of the nodule (Plate 7.4G). After 35 days of culture shoot bud formation was observed (Plate 7.4H) and a further ten days revealed the presence of root structures (Plate 7.4I).

Plate 7.4. Histological examination of regeneration from thin cell layers isolated from internodal stem segments of *S. chacoense* and cultured on medium containing 2,4-D and zeatin riboside

A. Periclinal divisions in the sub-epidermal region (A) has resulted in layers of flattened cells after four days of culture x 219

B. After seven days of culture regions of intense cellular activity were observed within the sub-epidermal layer x 437

C. A periclinal division (A) in the uppermost epidermal layer (L1) x 1093


A


B

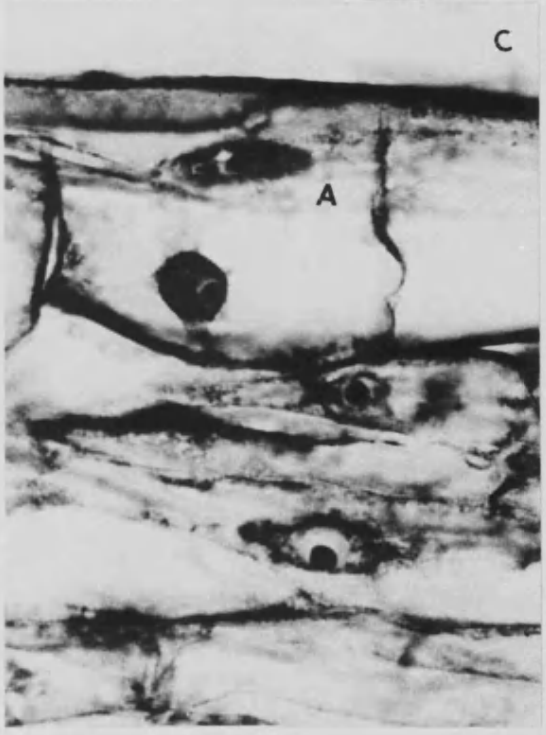

C
A

Plate 7.4. Histological examination of regeneration from thin cell layers isolated from internodal stem segments of *S. chacoense* and cultured on medium containing 2,4-D and zeatin riboside

D. After ten days of culture the majority of thin cell layer explants consisted of 15 to 20 cell layers X 44

E. After 14 days of culture areas of meristematic activity (A) were observed usually at the explant surface x 437

F. Formation of nodules (A) within the thin cell layer containing actively dividing and differentiating cells (B) x 219


D


E
A

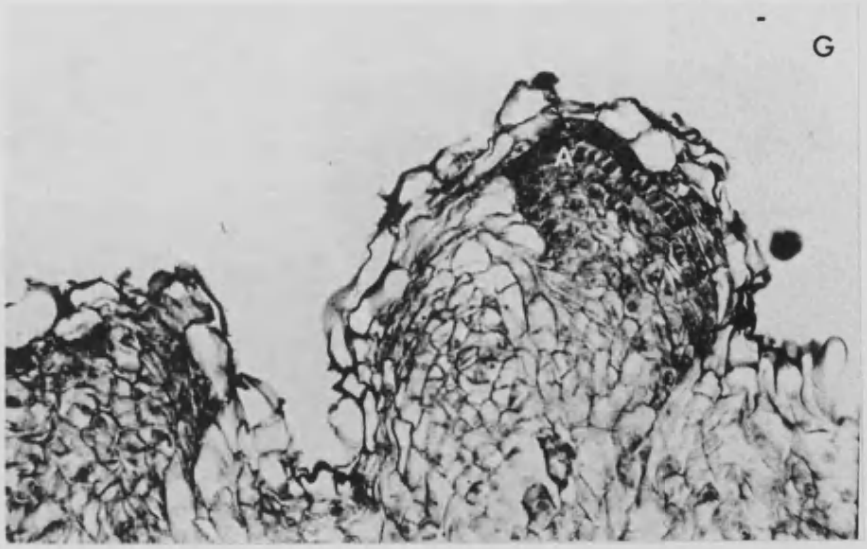

F
A
B
A
A
B

Plate 7.4. Histological examination of regeneration from thin cell layers isolated from internodal stem segments of *S. chacoense* and cultured on medium containing 2,4-D and zeatin riboside

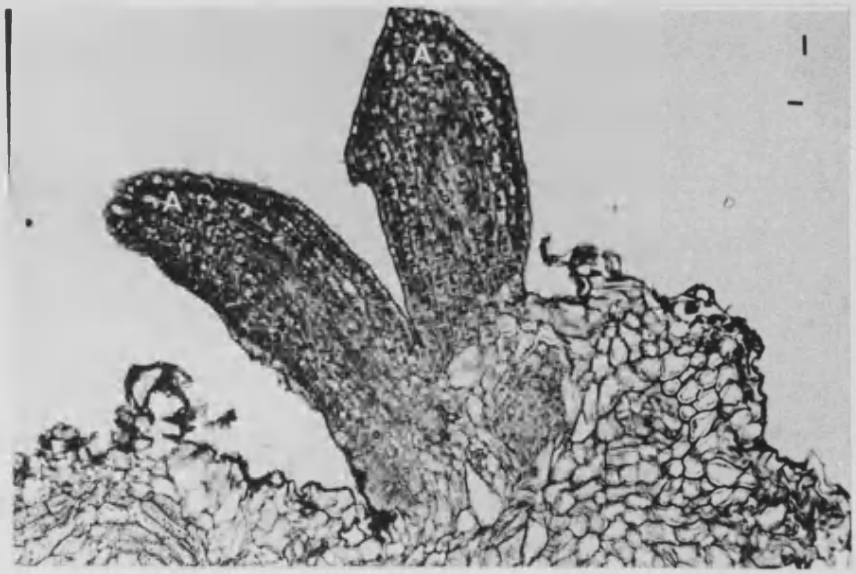
G. Zones of meristematic activity (A) were observed within the nodules (see Plate 7.4F) just beneath the surface of the nodule x 219

H. Shoot bud formation (A) at the surface of the explant after 35 days of culture. Vascular elements have also been formed (B) x 219

I. 45 day old explant showing the formation of root structures (A) x 219


G
A


H
A
B


I
A
A

7.7. MORPHOGENETIC RESPONSE OF THIN CELL LAYER GRAFTED INTERNODES

The aim of this experiment was to induce shoot regeneration from those thin cell layer grafted internodes where unions had been established between donor and recipient tissues of differing ploidy levels. If multiple shoot regeneration could be encouraged, it was considered conceivable that shoots composed of more than one genotype, that is, chimeral shoots would arise.

Thin cell layer grafted explants (heterografts) achieved through experiments described in Section 6.2.11 were transferred to a number of regeneration media. Prior to transfer, the strength of the graft union was determined as described in Section 6.2.2 and, in addition, any excess callus tissue at the ends of the explants was removed, in order to facilitate the identification of the origin (genotype) of any regenerating shoots.

A total of 233 thin cell layer grafted explants of mixed genotype, that is, heterografts were transferred to the following regeneration media: E31/E41; E32/E42 and 4ZR (for details of media, see Table 7.1, Section 7.2). Of these, 98 explants were of tetraploid donor tissue grafted onto diploid recipient tissue (4n→ 2n), and 135 of diploid donor tissue grafted onto tetraploid recipient tissue (2n → 4n). Only those explants achieving a strong graft union on 2.0NAA/2.0BAP medium (Section 6.2.11) were selected for this experiment. Explants were cultured under a light intensity of 85$\mu Mm^{-2}s^{-1}$PAR (16h daylength) and at a temperature of 22 ± 1°C and were examined regularly for signs of regeneration.

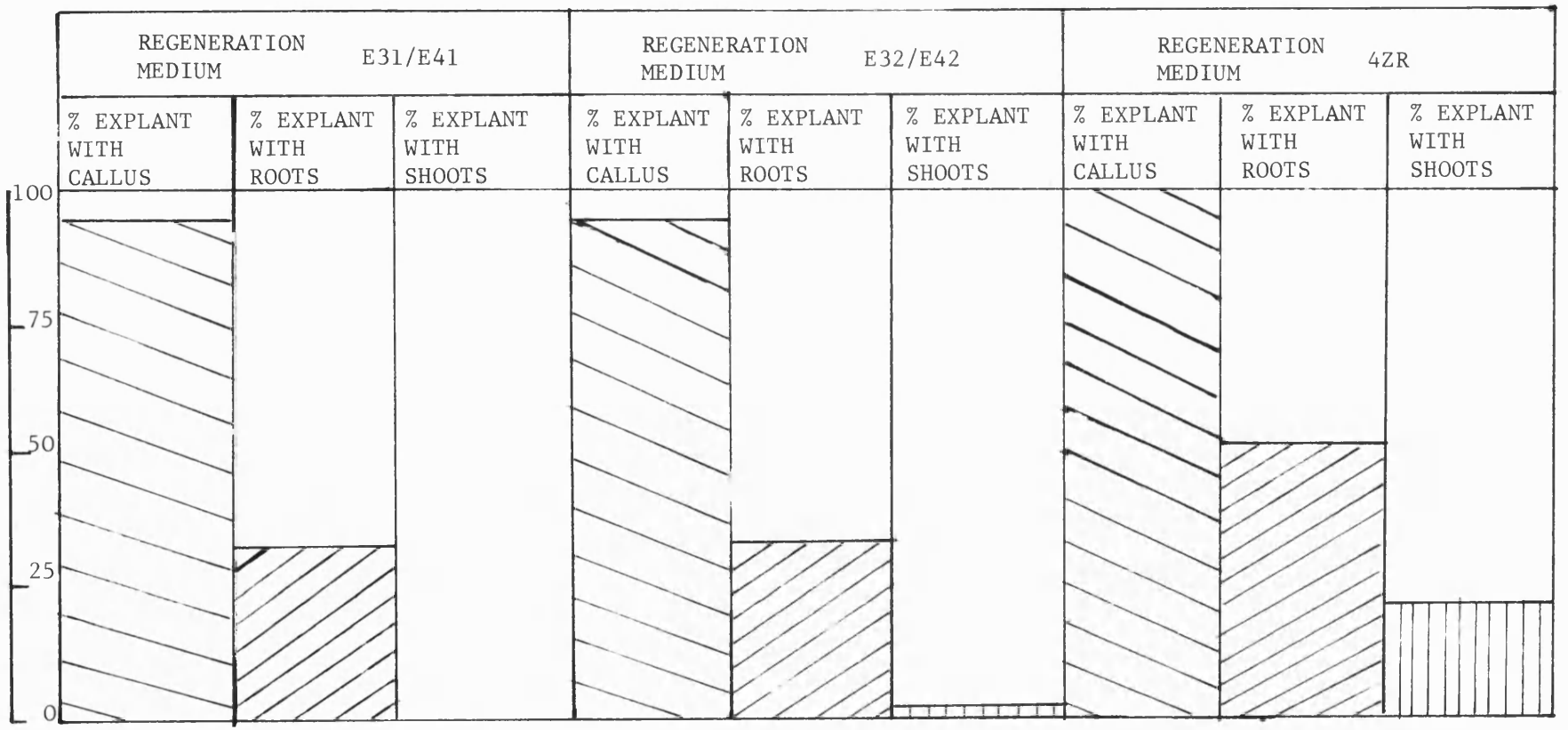
The majority of explants produced callus which varied in texture from dense and compact to open and friable: similarly the

colour of callus varied too, both within and between explants. A small proportion of explants became brown and desiccated (4%).

Root formation was the major type of regeneration observed over the culture period; 51% explants produced roots when cultured on 4ZR; 31% explants produced roots when cultured on E32/E42, and 30% explants produced roots when cultured on E31/E41 (Fig. 7.1).

Of the total number of explants cultured, only 16 produced shoots. 14 of these explants were cultured on 4ZR medium and two were cultured on E32/E42. 81% of these regenerating explants were composed of diploid donor tissue grafted onto tetraploid recipient tissue. Although the number of explants regenerating shoots was low, the major form of shoot production was multiple. From the 16 responsive explants a total of 161 shoot-tips were isolated and transferred to semi-solid basal culture medium supplemented with 4% sucrose (w/v). Shoot production occurred over a period of six months of culture on regeneration media, the shortest period of time prior to shoot regeneration being six weeks. However, these explants produced only single shoots (Plate 7.5A), whereas the most prolific shoot regeneration resulted when explants were in culture for periods of four to six months on media supplemented with zeatin riboside (Plates 7.5A, 7.5B, 7.5C, and 7.5D). In all cases, shoots have developed from callus produced from the grafted explant and it was not possible to distinguish the site of origin of this callus. The appearance of these regenerated shoots will be discussed in Chapter 8.

Fig. 7.1. Morphogenetic response of thin cell layer heterografts when cultured on three different regeneration media



Key (Fig. 7.1)

Media: MS + 2% sucrose (w/v) + various combinations of different growth regulators (see Table 7.1, Section 7.2)

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Period of culture: 6 months

Number of replicates per treatment: E31/E41 medium - 77

E32/E42 medium - 77

4ZR medium - 79

For actual figures see Appendix II.3.

Plate 7.5. Morphogenetic response of thin cell layer heterografts composed of S. sparsipilum (diploid) and S. tuberosum ssp. tuberosum cv. Pentland Ivory (tetraploid) after culture on a medium containing zeatin riboside ($4mgl^{-1}$)

A. Single shoot regeneration from a thin cell layer heterograft composed of diploid donor tissue and tetraploid recipient tissue after six weeks of culture x 10

B. Multiple shoot formation from a thin cell layer heterograft composed of diploid donor tissue and tetraploid recipient tissue after six months culture. Shoots have developed from the callus produced from the grafted explant x 6


A

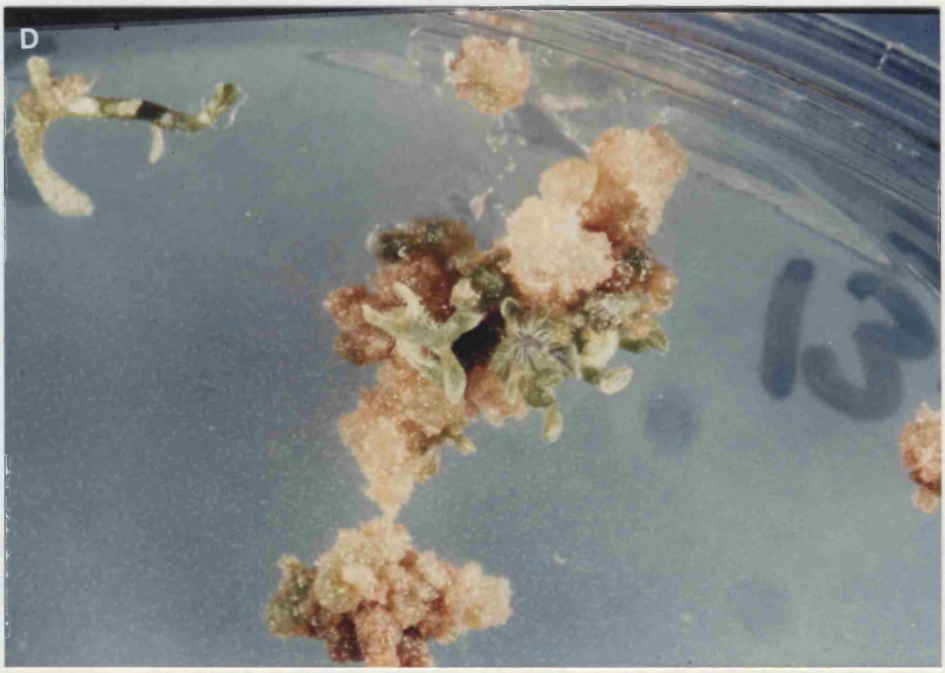

B

Plate 7.5. Morphogenetic response of thin cell layer heterografts composed of *S. sparsipilum* (diploid) and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory (tetraploid) after culture on a medium containing zeatin riboside ($4mgl^{-1}$)

C. As Plate 7.5B but shoots have appeared after four months of culture x 1

D. As Plate 7.5C but shoots have appeared after three months of culture


C


D

7.8. MORPHOGENETIC RESPONSE OF THIN-CELL LAYER GRAFTED EXPLANTS WHERE DONOR AND RECIPIENT TISSUE ARE OF THE SAME GENOTYPE

The aim of this experiment was to assess the influence grafted explants composed of tissue of differing ploidy level might have on the regeneration potential of that explant, through examining the regeneration potential of thin-cell layer autografts.

Thin-cell layer grafted explants (autografts) achieved by experimental procedure described in Section 6.2.13 were transferred to the same regeneration media as used in Section 7.7. Experimental procedure and culture conditions were as described in Section 7.7. For each medium treatment 40 autografts were cultured, 20 composed of tissue from S. sparsipilum and 20 composed of tissue from S. tuberosum ssp. tuberosum cv. Pentland Ivory.

The pattern of development resulting from culture on the three regeneration media was similar to that described for the heterograft explants in Section 7.7. Callus production was the main response observed, however, after six weeks of culture, 17% of all explants had developed roots and 12% had produced shoots. A further seven weeks of culture resulted in an increased production of both shoots and roots from all species and cultivars with cv. Pentland Ivory achieving the maximum shoot regeneration on 4ZR medium. Shoot regeneration was of the single shoot per explant form and there was a tendency for this single shoot to arise from the ends of the recipient partner of the graft (Plate 7.6A). However, with some explants, regenerating shoots were seen emerging through callus tissue in the area of the graft itself (Plate 7.6B). With both S. sparsipilum

and S. tuberosum ssp. tuberosum cv. Pentland Ivory, culture on 4ZR medium achieved maximum shoot and root production (Table 7.6).

TABLE 7.6. Organogenic response of thin cell layer grafted explants (autografts)

SPECIES/ CULTIVARS	REGENERATION MEDIA	% OF EXPLANTS PRODUCING SHOOTS	% OF EXPLANTS PRODUCING ROOTS
S. tuberosum ssp. tuberosum cv. Pentland Ivory	E31/E41	30	25
cv. Pentland Ivory	E32/E42	35	45
cv. Pentland Ivory	4ZR	70	60
S. sparsipilum	E31/E41	10	0
S. sparsipilum	E32/E42	10	0
S. sparsipilum	4ZR	50	50

Key:

Media: MS + 2% sucrose (w/v) + varying concentrations of different growth regulators (for details see Table 7.1 Section 7.2)

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Number of replicates per treatment: 40

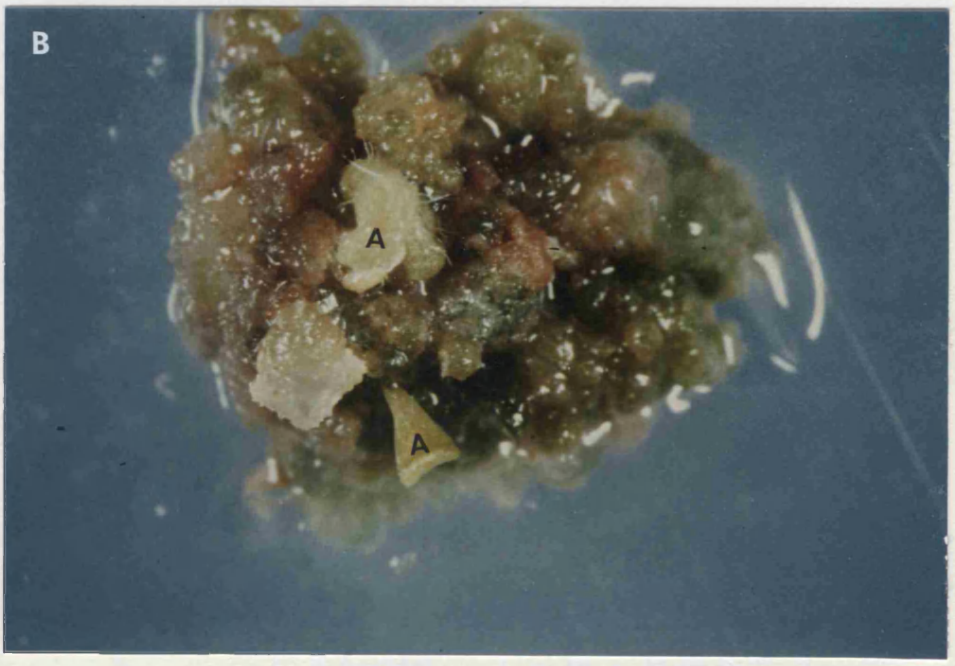
Period of culture: 6 months

Plate 7.6. Morphogenetic response of thin cell layer grafted explants where donor and recipient tissue are of the same genotype after culture on a medium containing zeatin riboside ($4mgl^{-1}$)

A. Single shoot regeneration from a thin cell layer autograft composed of tissue derived from S. tuberosum ssp. tuberosum cv. Pentland Ivory after 53 days of culture. This single shoot had developed from the ends of the recipient partner of the graft x 6

B. Shoot initiation from a thin cell layer autograft composed of tissue derived from S. tuberosum ssp. tuberosum cv. Pentland Ivory after 38 days of culture. Shoot development, (A) in this instance, was from the callus tissue in the area of the graft itself x 16


A


B
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A

7.9. THE INFLUENCE OF GROWTH REGULATORS IN THE GRAFTING MEDIUM ON MORPHOGENETIC COMPETENCE OF STEM EXPLANTS

In view of the limited regeneration achieved with both thin cell layer heterografts and autografts (as reported in Sections 7.7 and 7.8), it was decided to ascertain the effect that the growth regulators in the grafting medium have on regeneration potential of non-grafted stem internodal explants.

The experimental procedure was as described in Section 7.2. Other combinations of growth regulators were used in the grafting medium besides those found to encourage graft formation (Section 6.2.11), in order to compare results. The grafting media used consisted of basal culture medium (Section 2.2) supplemented with 3% sucrose (w/v) and the following growth regulators.

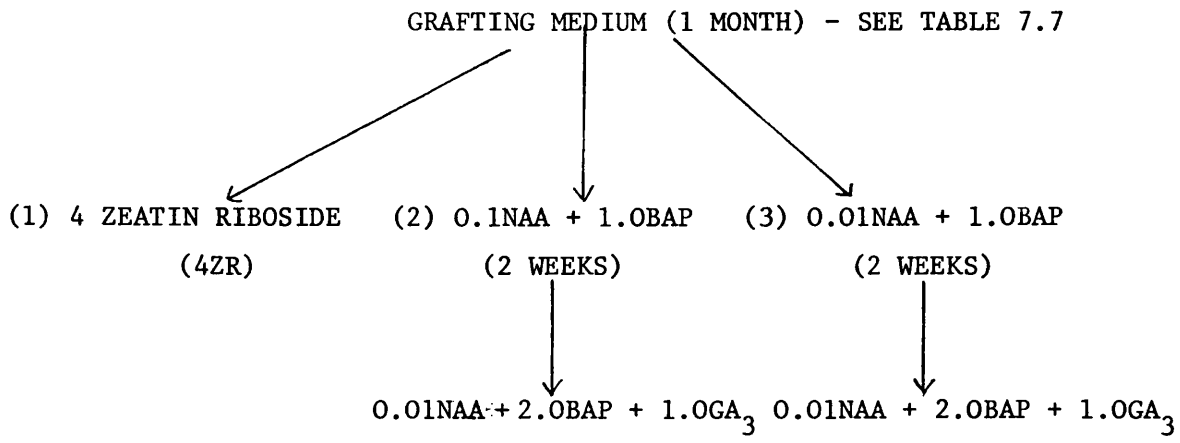
TABLE 7.7

MEDIA	AUXIN MGL^{-1}	CYTOKININ MGL^{-1}
1	2NAA	2BAP
2	2IAA	2BAP
3	2IAA	2KIN
4	1NAA	1BAP
5	1IAA	1BAP
6	0.2IAA	0.2KIN

Intact stem explants were cultured on the media listed in Table 7.7 for one month and then transferred to different media either by way of a one-stage or two-stage process as shown in Fig. 7.2. These media consisted of basal culture medium (Section 2.2)

supplemented with 3% sucrose (w/v) and the following combinations of growth regulators in mgl^{-1}.

FIG. 7.2



The species and cultivars used in this investigation were S. tuberosum ssp. tuberosum cv. Pentland Ivory and S. sparsipilum. Each treatment consisted of 40 replicates. Treatments were included where explants were cultured directly onto regeneration media.

As observed in previous experiments (Section 7.2) the ability of S. sparsipilum to produce shoots is maximised when cultured on media containing 4ZR. However, prior culture on a grafting medium appeared to affect that ability resulting in reduced shoot production (Table 7.8). Both root and shoot production did not attain the level reached with direct culture on 4ZR media. This affect was less noticeable when the grafting medium contained IAA instead of NAA; in this instance, shoot production was less affected than root production.

The regeneration potential of cv. Pentland Ivory was similarly affected by prior culture on grafting media, though to a lesser degree. Root production was more inhibited than shoot production when the regeneration medium used was 4ZR and conversely, shoot production was more severely affected when regeneration media (2) and (3) were used (Table 7.8).

The optimum combination of growth regulators for shoot production from both species and cultivars was 1.0IAA + 1.0BAP → 4ZR, however, the former medium does not induce an effective graft union. The results from this experiment are represented in histogram form in Fig. 7.3.

TABLE 7.8. Morphogenic responses of stem internodal explants on regeneration media after prior culture on a range of media containing various concentrations of growth regulators

SPECIES AND CULTIVAR	GRAFTING MEDIUM 2.0NAA + 2.0BAP MGL^{-1}						GRAFTING MEDIUM 1.0NAA + 1.0BAP MGL^{-1}					
	REGENERATION MEDIUM						REGENERATION MEDIUM					
	1		2		3		1		2		3	
	S	R	S	R	S	R	S	R	S	R	S	R
S. TUBEROSUM SSP. TUBEROSUM CV. PENTLAND IVORY	100	50	0	65	0	100	100	62	0	100	0	100
S. SPARS-IPILUM	0	0	0	60	0	30	0	0	0	65	0	25

SPECIES AND CULTIVAR	GRAFTING MEDIUM 2.0IAA + 2.0BAP MGL^{-1}						GRAFTING MEDIUM 1.0IAA + 1.0BAP MGL^{-1}					
	REGENERATION MEDIUM						REGENERATION MEDIUM					
	1		2		3		1		2		3	
	S	R	S	R	S	R	S	R	S	R	S	R
S. TUBEROSUM SSP. TUBEROSUM CV. PENTLAND IVORY	75	52	65	100	100	100	100	15	0	100	35	100
S. SPARS-IPILUM	42	22	0	0	0	0	57	50	0	0	0	0

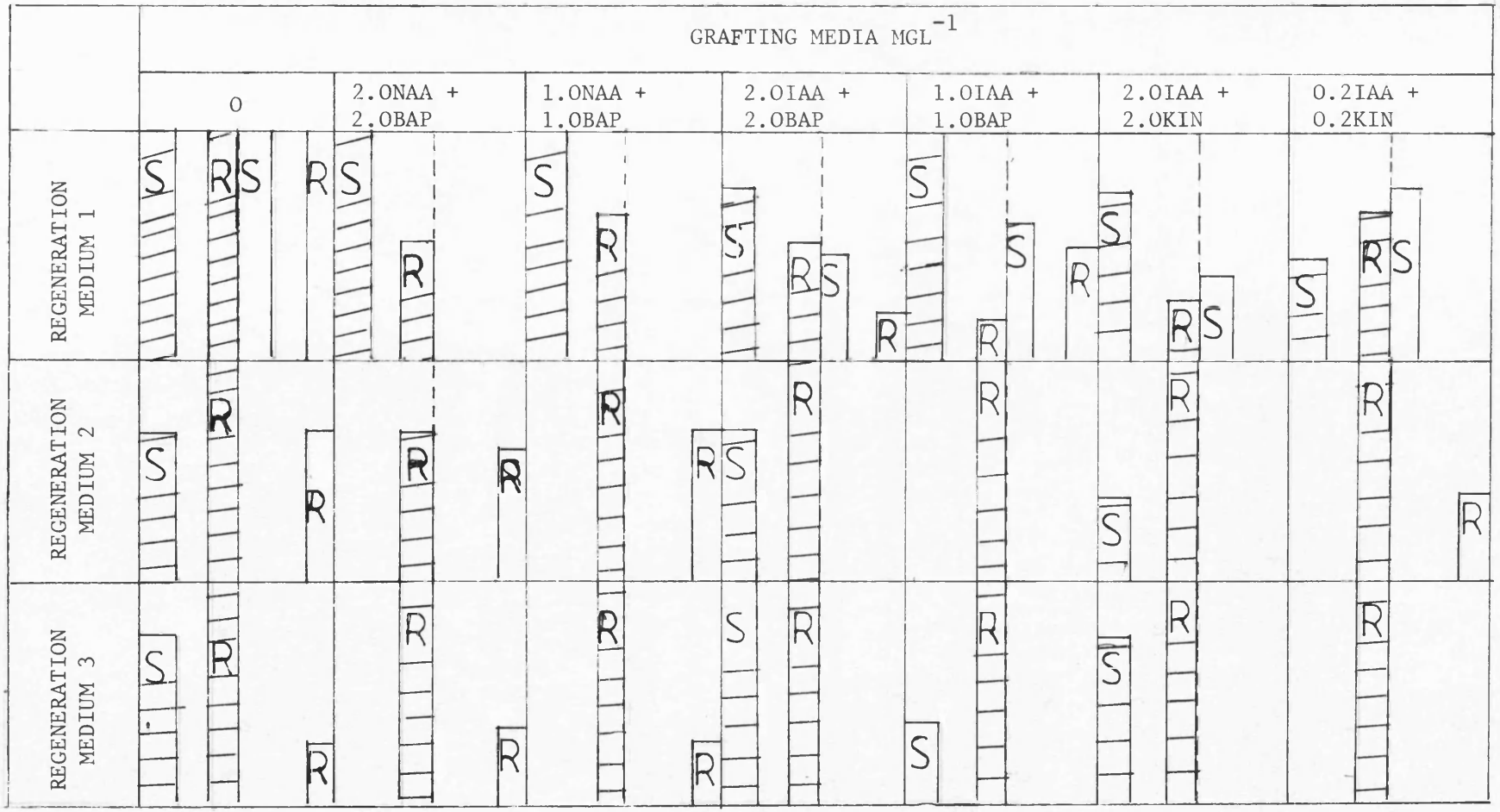
TABLE 7.8. CONTINUED

SPECIES AND CULTIVAR	GRAFTING MEDIUM 2.01AA + 2.0KIN MGL^{-1}						GRAFTING MEDIUM 0.21AA + 0.2KIN MGL^{-1}					
	REGENERATION MEDIUM						REGENERATION MEDIUM					
	1		2		3		1		2		3	
	S	R	S	R	S	R	S	R	S	R	S	R
S. TUBEROSUM SSP. TUBEROSUM CV. PENTLAND IVORY	70	32	35	100	65	100	37	55	0	100	0	100
S. SPARS-IPILUM	35	0	0	0	0	0	67	0	0	30	0	0

SPECIES AND CULTIVAR	NO PRECULTURE ON A GRAFTING MEDIUM					
	REGENERATION MEDIA					
	1		2		3	
	S	R	S	R	S	R
S. TUBEROSUM SSP. TUBEROSUM CV. PENTLAND IVORY	100	100	65	100	75	100
S. SPARS-IPILUM	100	100	0	57	0	25

Fig. 7.3

Morphogenic responses of stem internodal explants on regeneration media after prior culture on a range of media containing various concentrations of growth regulators



Key (for Table 7.8 and Fig. 7.3)

Media: Grafting media: MS + 3% sucrose (w/v) + growth regulators as detailed in Table 7.7

Regeneration media: MS + 3% sucrose (w/v) + combinations of growth regulators as detailed in Fig. 7.2.

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Number of explants per treatment: 40

Period of culture: Grafting medium - 4 weeks

Regeneration medium - 13 weeks

S: % explants producing shoots

R: % explants producing roots

Height of bars: % of explants showing particular morphogenic response

S: shoot production

R: root production

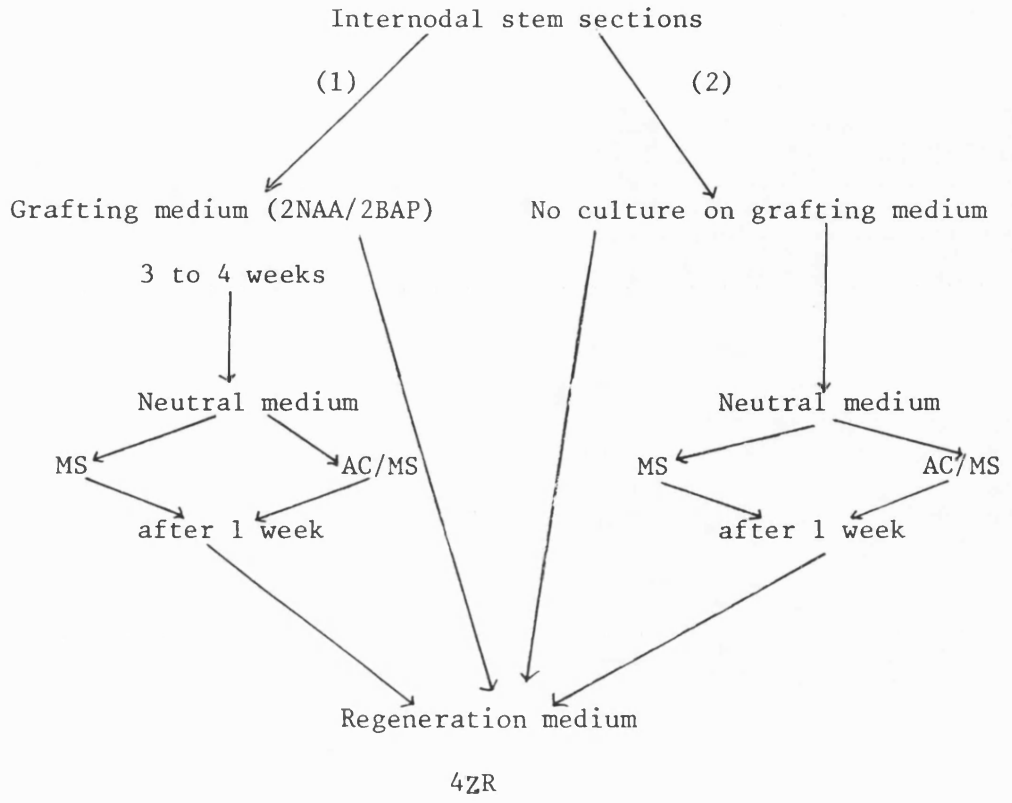
▨ : S. tuberosum ssp. tuberosum cv. Pentland Ivory

□ : S. sparsipilum

7.10. INFLUENCE OF A TWO-STEP PROCEDURE ON REGENERATION OF THIN CELL LAYER GRAFTED EXPLANTS

It was intended that this investigation should assess the importance of interrupting the established procedure of culturing explants on grafting medium for a period of three to four weeks followed by culture on regeneration medium (4ZR), with a period of culture on a "neutral" medium. Such a medium might rectify the balance of endogenous growth regulators, seemingly affected by the grafting process, so that the original regeneration potential of the explants would be restored. Grafted material was prepared as described in Section 6.2.11 from tissue derived from S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory. Internodal stem sections were obtained as reported in Section 7.2 from plantlets of the same species and cultivars as those participating in graft formation. All source material had been in culture for three to four weeks. Internodal stem sections, once excised from plantlets, were cultured in one of six ways, as outlined in Fig. 7.4. Details of grafting media (2NAA/2BAP) and regeneration medium (4ZR) were given in Sections 6.2.10 and 7.2 respectively. Two neutral media were employed, namely, basal culture medium (Section 2.2) supplemented with 3% sucrose (w/v) either with (AC/MS) or without the addition of 1% activated charcoal (MS). Explants, both grafted and non-grafted were cultured on these media for one week (see Fig. 7.4).

Fig. 7.4



Grafted explants, once graft unions were established, proceeded as indicated by the left-hand side (pathway one) of Fig. 7.4. For each treatment involving internodal stem sections, 40 explants were cultured, whereas grafted explants numbered 96 in total. Light and temperature conditions were as described in Section 7.7.

After one month on grafting medium (2NAA/2BAP), 58% of the total number of grafted explants had achieved strong, graft unions and 44% of these successful heterografts had produced roots. The intact internodal stem explants had undergone expansion and callus production to varying degrees as a result of culture on the grafting medium. In addition, root production had occurred from both

S. tuberosum ssp. tuberosum cv. Pentland Ivory and S. sparsipilum: 64% of explants derived from cv. Pentland Ivory and 6% of explants derived from S. sparsipilum had produced roots. The results of this experiment, after transferance to regeneration medium, were assessed over a period of 90 days.

Table 7.9 shows the results obtained in this experiment.

Culture of intact internodal stem segments derived from S. sparsipilum on grafting medium (2NAA/2BAP) resulted in a total inhibition of the organogenic process when the explants were eventually cultured on the regeneration medium (4ZR): shoot and root production being reduced from 100% and 87% to 0%. Culture on AC/MS or MS prior to culture on the regeneration medium (4ZR) did not improve regeneration except for a marginal increase of 5% for both root and shoot production when MS was the intervening "neutral" medium. When internodal stem segments derived from S. sparsipilum were cultured directly onto AC/MS or MS (without any prior culture on the grafting medium) both shoot and root production were severely inhibited when explants were eventually cultured on the regeneration medium (4ZR).

Culture of internodal stem segments derived from cv. Pentland Ivory on grafting medium (2NAA/2BAP mgl^{-1}) resulted in reduced root regeneration (from 70% to 50%), however, shoot production was seemingly not affected. Culture on AC/MS and MS prior to culture on the regeneration medium (4ZR) of these explants which had experienced culture on the grafting medium, led to decreased shoot production and increased root production. This was the

converse to the response observed when the explants had not been exposed to any period of culture on the grafting medium, where both shoot and root production was restricted, but the latter more so than the former.

Of the paths illustrated in Fig. 7.4, the grafted explants displayed optimum regeneration response when cultured on AC/MS medium prior to 4ZR regeneration medium.

TABLE 7.9. Influence of a two-step procedure on the organogenic potential of thin cell layer heterografts and intact stem internodal explants

INTERNODAL STEM EXPLANTS: NO PRIOR CULTURE ON GRAFTING MEDIUM						
SPECIES/ CULTIVARS	→4ZR		→AC/MS→4ZR		→MS→4ZR	
	SHOOTS	ROOTS	SHOOTS	ROOTS	SHOOTS	ROOTS
S. tuberosum ssp. tuberosum cv. Pentland Ivory	100	70	70	5	60	2
S. sparsipilum	100	87	45	0	17	10

INTERNODAL STEM EXPLANTS: PRIOR CULTURE ON GRAFTING MEDIUM						
SPECIES/ CULTIVARS	→4ZR		→AC/MS→4ZR		→MS→4ZR	
	SHOOTS	ROOTS	SHOOTS	ROOTS	SHOOTS	ROOTS
S. tuberosum ssp. tuberosum cv. Pentland Ivory	100	50	15	70	0	95
S. sparsipilum	0	0	0	0	5	5

TABLE 7.9. CONTINUED

THIN CELL LAYER HETEROGRAFTS CULTURED ON GRAFTING MEDIUM						
TYPE OF GRAFT	→4ZR		→AC/MS→4ZR		→MS→4ZR	
	SHOOTS	ROOTS	SHOOTS	ROOTS	SHOOTS	ROOTS
2n→4n	37	10	65	65	22	37
4n→2n	0	0	0	0	0	12

Key:

Figures represent % of explants producing shoots and roots.

Media: grafting medium; 2.0NAA/2.0BAP mgl^{-1} (3 to 4 weeks)

AC/MS; see Section 7.11 (1 week)

MS; see Section 7.11 (1 week)

regeneration medium; MS + 2% sucrose (w/v) + 4.0mgl^{-1} zeatin riboside (90 days)

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Number of replicates per treatment: Internodal stem explants; 40

Thin cell layer heterografts with strong graft unions; 56

Graft type: 2n→4n; diploid donor tissue grafted onto tetraploid recipient tissue

4n→2n; tetraploid donor tissue grafted onto diploid recipient tissue

7.11. HISTOLOGICAL EXAMINATION OF REGENERATION FROM THIN CELL LAYER GRAFTED EXPLANTS

Histological examination of thin cell layer grafted explants aimed to ascertain the involvement of both donor and recipient tissue in shoot formation. However, this proved difficult mainly due to the problems experienced in identifying donor and recipient tissue in long established thin cell layer grafted explants, and because shoots often were produced after periods of time, far exceeding that allowed for in this investigation. Some attempt, however, was made at evaluating the possibility of chimeral shoot formation, wherever possible.

Thin cell layer grafted explants were prepared as described in Section 6.2.11 and then cultured on regeneration media 4ZR as described in Section 7.7. At various intervals, explants were taken out of culture and prepared for histological examination (Section 2.5).

Examination of sections through these explants after six days revealed areas of the graft where donor and recipient tissue were in direct contact (Plate 7.7A). Furthermore, these areas of contacting tissue were seen to be sited in positions shown by previous experiments on regeneration (Section 7.3.2) to favour shoot regeneration. The cells in these areas were active, although the activity was of a disorganized nature. Further histological observations of the thin cell layer grafted explants revealed meristematic activity occurring at different times and in different locations throughout the grafted explant. Meristematic activity,

possibly indicative of early shoot formation, was observed where it seemed only the cells of the donor tissue were involved in production of that organized tissue (Plate 7.7B). Similarly, possible shoot primordia were seen developing from callus tissue which had arisen from the base of the recipient tissue of the graft (Plate 7.7C). Areas of seemingly organized cell activity were often revealed within the callus bridge of the graft (Plate 7.7D), and consequently, the genotypic nature of the cells contributing to these organized groups of cells was indeterminable.

After 20 days on regeneration media, many explants had developed several meristematic areas arising from the recipient tissue (Plate 7.7E). 30 days of culture revealed further meristematic activity. This either took the form of what appeared to be root formation originating from the original vascular region of the recipient tissue (Plate 7.7F), or less well-defined areas of meristematic activity, which like Plate 7.7B seemingly involved cells of the donor tissue alone. No thin cell layer grafted explants were sectioned which allowed positive identification of donor and recipient tissue in any potential shoot formation.

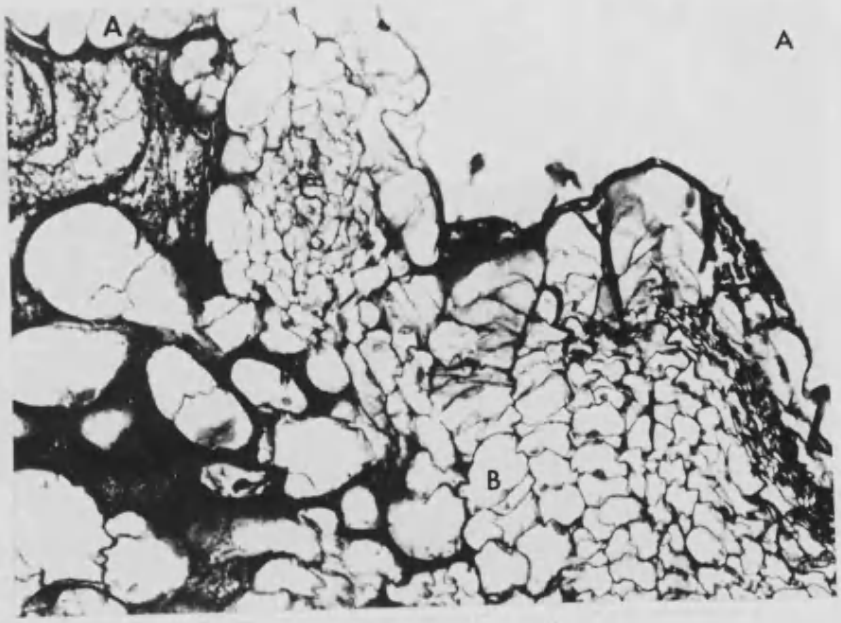
Plate 7.7

Histological examination of regeneration from thin cell layer heterografts between S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory cultured on medium containing zeatin riboside ($4mgl^{-1}$). The heterografts consisted of diploid tissue grafted onto tetraploid tissue.

A. Contact established (C) between the donor component (A) of the heterograft and the recipient component (B). Cells in this area (C) are active, however there is little indication of the cellular organization necessary for shoot initiation after six days of culture on regeneration medium x 219

B. Meristematic activity, possibly indicative of early shoot formation (A) observed in a 20 day old explant. It would appear that only the cells of the donor component (B) of the heterograft are involved in the production of this organized tissue x 109

C. The development of a possible shoot primordium (A) from the callus tissue (B) which had arisen from the base of the recipient component (C) of the graft after 20 days of culture x 109


A
A
B


B
A
B
C

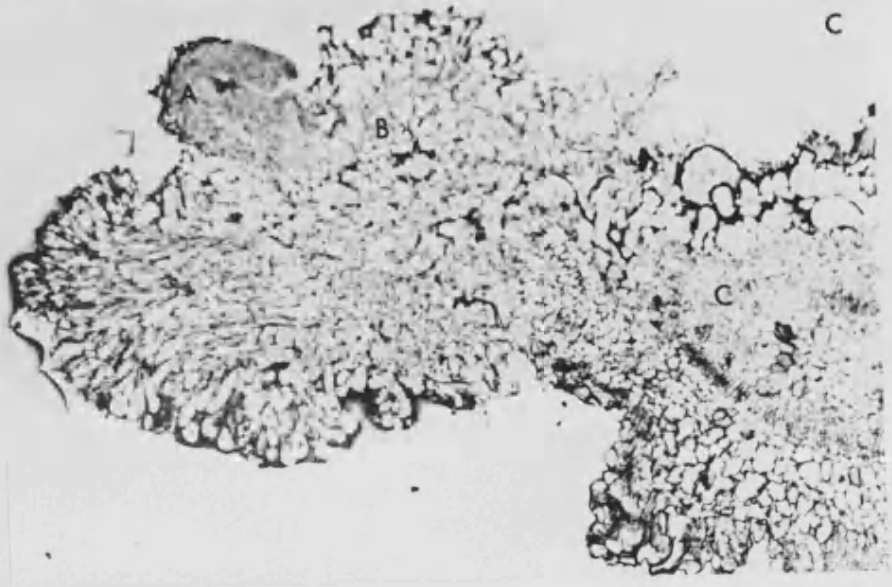

C
A
B
C

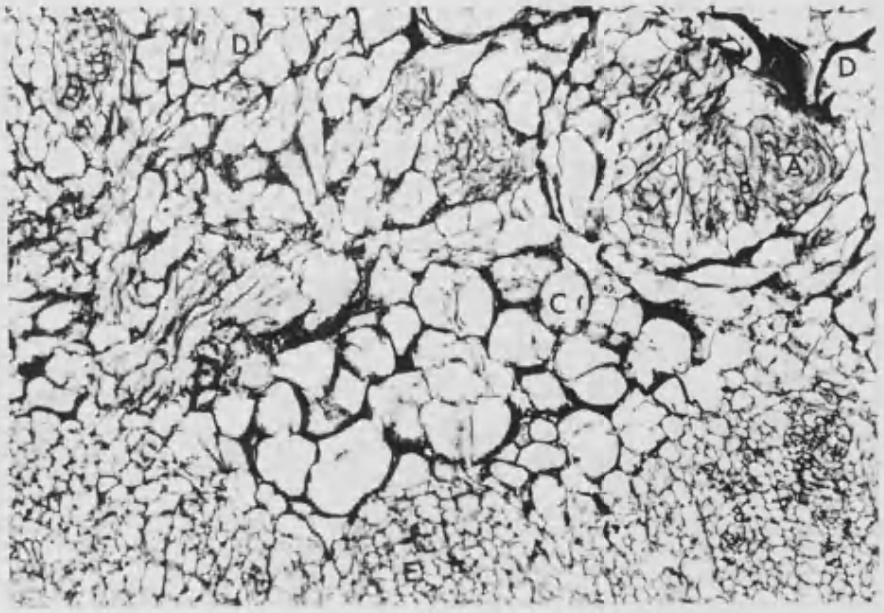
Plate 7.7

Histological examination of regeneration from thin cell layer heterografts between S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory cultured on medium containing zeatin riboside ($4mgl^{-1}$). The heterografts consisted of diploid tissue grafted onto tetraploid tissue.

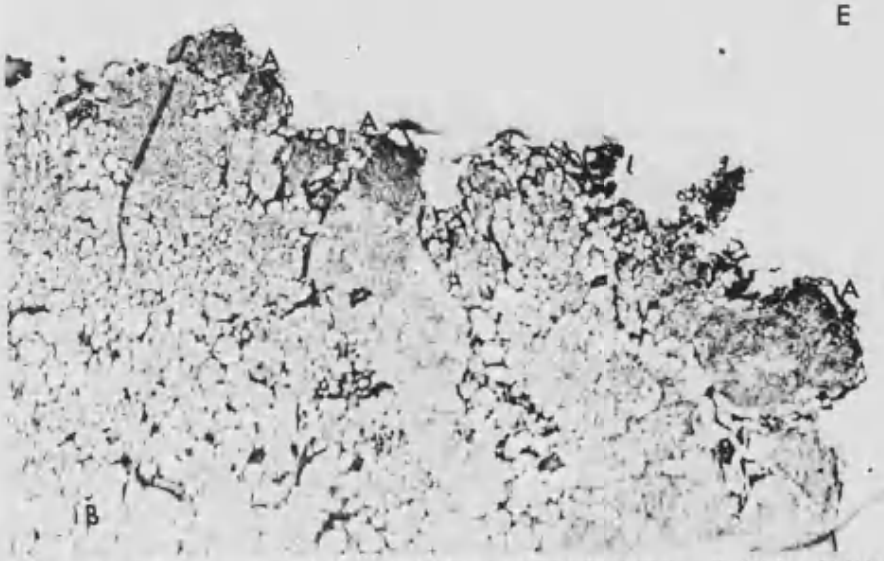
D. Areas of cellular activity (A) showing the formation of vascular elements (B) within the callus bridge (C) which separates the donor component (D) of the heterograft from the recipient component (E) x 219

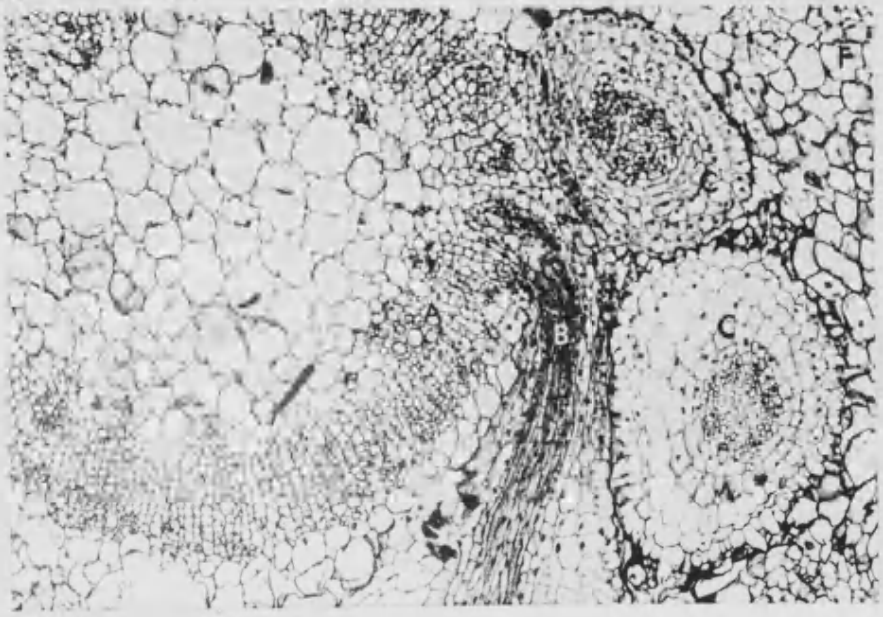
E. Regions of meristematic activity (A) arising from the callus generated by the recipient component (B) of the heterograft after 20 days of culture x 109

F. 30 day old explant showing root formation (B) originating from the original vascular tissue (A) of the recipient component of the heterograft. Meristematic nodules (C) have been formed in the region external to the original vascular tissue (A) x 109



E





7.12. REGENERATION OF CALLUS CULTURES

It was intended that this investigation should demonstrate, which medium out of a range of media investigated, was capable of encouraging shoot production from callus of single species (Section 4.2).

Callus cultures were obtained as described in Section 4.2. Only those explants of healthy appearance and displaying signs of active growth were transferred to regeneration media. Transferance of these callus cultures to regeneration media occurred at the end of a subculture period, that is, three to four weeks after subculturing onto fresh callus medium. Age of callus cultures on transfer to regeneration media was four months. All callus explants had been cultured on media containing $2.0 mgl^{-1}$ 2,4-D except those derived from S. brevidens, where the callus induction and proliferation medium contained NAA ($2.0 mgl^{-1}$).

Regeneration media used, consisted of basal culture medium (Section 2.2) supplemented with 2% sucrose (w/v) and various combinations of growth regulators (Table 7.10). The choice of media was influenced by personal experience with regeneration studies of stem explants, and reports of successful callus regeneration media in the literature.

TABLE 7.10

REGENERATION MEDIUM	AUXIN MGL^{-1}	CYTOKININ MGL^{-1}	GIBBERELLIN MGL^{-1}
1K (Wang et al., 1975)	-	1.0 kinetin	-
10K Wang et al., 1975)	-	10.0 kinetin	-
.4B (Lam, 1975)	-	0.4 BAP	-
M1 (Roest et al., 1976)	1.0 1AA	1.0 BAP	10.0 GA_3
M2 (Marani et al., 1977)	-	1.0 BAP	1.0 GA_3
4ZR	-	4.0 zeatin riboside	-

E31/E41 and E32/E42 were also used in this investigation, as a two-stage procedure, with a period of five to seven days separating the two stages (see Section 7.2, Table 7.1).

Species and cultivars used in this investigation were as outlined in Section 4.2. Number of callus explants for each medium treatment was 40. Light and temperature conditions were as described in Section 7.7. Assessment of regeneration was made after periods of culture of one, two and four months.

The morphogenetic responses of the cultures are shown in Table 7.11. A proportion of explants had developed roots after one month of culture, however, after two months of culture, maximum

root development had occurred and there was no increase in root formation after four months of culture. Media M1 and M2 (Table 7.10) encouraged rhizogenesis in the maximum number of species and cultivars. The only shoot development was obtained from S. chacoense cultured on 4ZR (20%); a medium capable of inducing 100% shoot regeneration from stem explants of S. chacoense (Table 7.2, Section 7.2).

TABLE 7.11. Morphogenetic responses of callus derived from different species and cultivars of potato when cultured on various regeneration media

SPECIES/ CULTIVAR	MEDIA	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS
S. tuberosum	M1	-	20
ssp. tuberosum	M2	-	40
cv. Fortyfold	1K	-	47
cv. Fortyfold	10K	-	40
cv. Fortyfold	4ZR	-	60
cv. Fortyfold	E31/E41	-	-
cv. Fortyfold	E32/E42	-	40
cv. Fortyfold	.4B	-	27
S. tuberosum	M1	-	32
ssp. tuberosum	M2	-	-
cv. Majestic	1K	-	-
cv. Majestic	10K	-	-
cv. Majestic	4ZR	-	-
cv. Majestic	E31/E41	-	-
cv. Majestic	E32/E42	-	-
cv. Majestic	.4B	-	-

TABLE 7.11. CONTINUED

SPECIES/ CULTIVAR	MEDIA	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS
S. tuberosum	M1	-	40
ssp. tuberosum	M2	-	60
cv. Pentland Squire	1K	-	27
cv. Pentland Squire	10K	-	40
cv. Pentland Squire	4ZR	-	-
cv. Pentland Squire	E31/E41	-	-
cv. Pentland Squire	E32/E42	-	-
cv. Pentland Squire	.4B	-	-
S. tuberosum	M1	-	60
ssp. tuberosum	M2	-	80
cv. Pentland Ivory	1K	-	-
cv. Pentland Ivory	10K	-	47
cv. Pentland Ivory	4ZR	-	-
S. tuberosum	E31/E41	-	40
ssp. tuberosum	E32/E42	-	-
cv. Pentland Ivory	.4B	-	20

TABLE 7.11. CONTINUED

SPECIES/ CULTIVAR	MEDIA	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS
S. sparsipilum	M1	-	-
S. sparsipilum	M2	-	-
S. sparsipilum	1K	-	27
S. sparsipilum	10K	-	-
S. sparsipilum	4ZR	-	-
S. sparsipilum	E31/E41	-	-
S. sparsipilum	E32/E42	-	-
S. sparsipilum	.4B	-	-
S. chacoense	M1	-	20
S. chacoense	M2	-	-
S. chacoense	1K	-	-
S. chacoense	10K	-	40
S. chacoense	4ZR	20	40
S. chacoense	E31/E41	-	-
S. chacoense	E32/E42	-	40
S. chacoense	.4B	-	47

TABLE 7.11. CONTINUED

SPECIES/ CULTIVAR	MEDIA	% EXPLANTS FORMING SHOOTS	% EXPLANTS FORMING ROOTS
S. brevidens	M1	-	-
S. brevidens	M2	-	-
S. brevidens	1K	-	-
S. brevidens	10K	-	-
S. brevidens	4ZR	-	-
S. brevidens	E31/E41	-	-
S. brevidens	E32/E42	-	-
S. brevidens	.4B	-	-

Key:

Media: MS + 2% sucrose + various combinations of growth regulators as detailed in Table 7.10

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Period of culture: 4 months

Number of replicates per treatment: 40

7.13. REGENERATION OF CALLUS CULTURES USING PROTOPLAST REGENERATION MEDIA

Because of the failure in achieving shoot regeneration using the media described in Section 7.12 it was decided to transfer single species callus induced as a result of procedures described in Sections 4.2 and 4.3 and callus explants of mixed species (Section 5.2), in culture for four months to different media to those employed in Section 7.12. The media chosen had induced shoot morphogenesis from protoplast callus derived from potato tissue, and was basically that of Shepard (1980) but with modifications recommended by Nelson et al., (1983). This recommendation led to the use of a two-stage procedure, D1 and D2, in which the first medium contained NAA ($0.1mgl^{-1}$) and BAP ($0.5mgl^{-1}$), and the second medium contained IAA ($0.1mgl^{-1}$) and zeatin ($1.0mgl^{-1}$). The other medium used was a modication of a procedure used by Schumann et al., (1980) and the growth regulators present were IAA ($0.1mgl^{-1}$), zeatin ($2.0mgl^{-1}$) and GA_3 ($0.1mgl^{-1}$). These media are fully described in Appendices I.3 and I.4.

Culture procedure and conditions were as described in Section 7.12. Tetraploid species and cultivars involved in this investigation were S. tuberosum ssp. tuberosum cv. Pentland Ivory, cv. Pentland Squire and cv. Fortyfold, the diploid species used were S. sparsipilum and S. chacoense. Both the single species and mixed species callus explants had been induced using three different media, namely $2.0mgl^{-1}$ 2,4-D (Section 4.2) and SCH and ST (Section 4.3). From each of these treatments the regeneration potential of 40 explants was examined in this experiment. Mixed

callus cultures took the form of OT and SS (Section 5.2).

After 70 days of culture, a number of mixed callus explants were showing visible signs of chlorophyll synthesis, however, this appeared to be a characteristic associated with the individual explant as cultures of the same origin subject to the same cultural conditions were responding differently on the same regeneration medium.

Table 7.12 shows the data resulting from the experiment, but only notes those cultures where regeneration of any form occurred. Regeneration only occurred when the two-stage procedure of D1/D2 was used, and only from those explants originally cultured on media containing 2,4-D ($2.0 mgl^{-1}$). Shoots and roots were produced by a large number of callus explants derived from a single species, but was more prevalent when the callus had been derived from a tetraploid species. Mixed callus cultures of both types were found to possess regenerative potential, the favoured combination being S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory (SS). Generally shoot regeneration took the form of single shoots, but two mixed callus cultures (S. sparsipilum + cv. Pentland Ivory - SS) gave rise to five and twenty shoots respectively. All shoots were isolated and transferred to rooting medium (Section 2.5). The morphology of shoots isolated from mixed callus cultures is described in Chapter 8.

TABLE 7.12. Morphogenetic responses of callus derived from different species and cultivars of potato when cultured on protoplast regeneration media

SPECIES/ CULTIVARS	% EXPLANTS PRODUCING SHOOTS	% EXPLANTS PRODUCING ROOTS
SINGLE SPECIES CALLUS		
S. tuberosum ssp. *tuberosum*		
cv. Pentland Ivory	70	40
cv. Pentland Squire	67	25
cv. Fortyfold	55	30
S. sparsipilum	20	15
S. chacoense	7	5
MIXED SPECIES CALLUS		
cv. Fortyfold + *S. sparsipilum* (SS)	25	-
cv. Pentland Ivory + *S. sparsipilum* (SS)	50	25
cv. Pentland Ivory + *S. sparsipilum* (OT)	37	12
cv. Pentland Squire + *S. sparsipilum* (OT)	12	-

Key:

Regeneration media: D1/D2 (Appendix 1.3)

SCH (Appendix 1.4)

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}s^{-1}$PAR

Callus induction and proliferation media:

2.0 2,4-D (mgl^{-1})(Section 4.2)

SCH/C(Section 4.3)

ST (Section 4.3)

Number of replicates per treatment:

Single species callus = 4o

Mixed species callus = 40

Period of culture on callus media:

4 months

Period of culture on regeneration media:

4 to 6 months

7.14. DISCUSSION

7.14.1. Regeneration from internodal stem segments

As discussed in Section 1.4.2 the cultural requirements necessary to induce shoot regeneration from the stem tissue of potato varies widely according to species and cultivar. Results obtained in this investigation would seem to imply a preference for zeatin and/or zeatin riboside in the regeneration medium with the species and cultivars tested, compared with other cytokinins such as kinetin and BAP. Maximum shoot and root regeneration was obtained for all species and cultivars when explants were cultured on a medium containing $4mgl^{-1}$ zeatin riboside. There appear to be no reports in the literature where zeatin riboside has been used as the sole growth regulator in the medium to achieve shoot and root regeneration in potato. Some workers (Austin *et al.*, 1983; Schumann *et al.*, 1980) have found zeatin to be a beneficial addition to regeneration media. Austin *et al.*, (1983) achieved regeneration from individual callus produced from separated potato stem callus cells when zeatin was used in the medium in combination with IAA. Similarly, Schumann *et al.*, (1980) used zeatin in combination with other growth regulators to induce shoots from both long-term callus cultures and protoplast callus derived from internodal tissue of *Solanum phureja*; the development of shoot primordia into shoots occurred only on media containing high levels of zeatin (2-5ppm). On the other hand, Wheeler *et al.*, (1985) substituted zeatin for BAP in the medium and found that shoot production improved only slightly for cv. Record, but the substitution had little effect on the other 14 cultivars tested.

The classic finding of Skoog et al., (1957) of the need for a balance between auxin and cytokinin in order for organogenic competence to be expressed, has been found to be necessary in many other plant species (Murashige, 1974; Evans et al., 1981). The balance tends to take the form of high cytokinin to auxin ratio favouring shoots, whereas the reverse ratio would favour root production. A review of the literature indicates that potato tissue responds to this type of growth regulator manipulation, and regardless of whether the induction procedure is one-stage or two-stage, the level of cytokinin always exceeds that of the auxin present for shoot production to occur (Westcott et al., 1977; Webb et al., 1983; Wheeler et al., 1985). The results achieved in this investigation also imply that relatively high concentrations of cytokinin (Section 7.2) are required to induce shoot formation in potato tissue. This need for cytokinin possibly reflects that endogenous levels of auxin are high in potato tissue.

Differences between the diploid and tetraploid species were evident when the two-stage procedures, E31/E41 and E32/E42 were used to encourage shoot and root regeneration from potato stem tissue. The tetraploid species responded to the media used in these procedures, producing roots and shoots from the cultured tissue, in contrast to the stem tissue derived from the diploid species which expressed little or no organogenic potential. The exception to this rule was S. tuberosum ssp. tuberosum cv. Fortyfold whose response to E31/E41 and E32/E42 procedures resembled that of a diploid species rather than a tetraploid

species (Section 7.2). E31/E41 and E32/E42 are both two-stage procedures where the stage two medium lacks the 2,4-D ($0.2mgl^{-1}$) present in the stage one medium. It is possible, therefore, that the diploid species are more sensitive to 2,4-D and that its presence in the initial medium is sufficient to inhibit shoot regeneration. This sensitivity could result from the higher endogenous levels of auxin in the diploid species compared with the tetraploid species, thus the addition of 2,4-D in the medium would prevent the adjustment of the auxin-cytokinin ratio neeessary to induce shoot production. Alternatively the relatively low levels of zeatin and zeatin riboside in these regeneration media ($2.0mgl^{-1}$ compared with $4.0\ mgl^{-1}$ zeatin riboside in the 4ZR medium) could be too low to provide the stimulus required to encourage shoot regeneration; this could also be indicative of high endogenous auxin concentrations in the diploid species. There have been many reports in the literature concerning genotype-dependent differences in the ease of plant regeneration, and it is now well accepted that genetic factors contribute to the response of plant tissues in culture (Dunwell, 1981; Raquin, 1982). Potato is no exception, as is shown by the work of Wheeler et al., (1985) where the regeneration potential of 14 cultivars of S. tuberosum L were widely different. In addition, the extensive range of media reported in the literature as favouring regeneration in potato tissue serves as further evidence for the individuality that can exist between cultivars and species.

In this investigation light was shown to be an important factor in the induction of shoots and roots. Root regeneration markedly increased when daylength was changed from a five hour period to a 16 hour period; shoot production also increased but the effect was less evident and varied with species and cultivar. Increasing the light intensity from 20μMm^{-2}s^{-1}PAR to 85μMm^{-2}s^{-1}PAR had a less marked effect on regeneration, however, there was a general tendency for shoot and root regeneration to improve under the higher light intensity (Section 7.2). Ammirato (1986) reports on the strong morphogenic effect light can exert on plant development in culture and provides several examples of plant species where light is an important factor in promoting initiation and growth of shoots. Hussey et al., (1981) investigated the effect of light on in vitro growth of shoots of several cultivars of S. tuberosum L. and found that the daylength, but not light intensity, to be a critical factor in determining the morphology of the shoot. The results from this investigation would appear to show that daylength exerts a strong influence on regeneration in the species and cultivars of potato tested.

As wounding was a necessary part of the techniques used in this study, it was considered important to look closely at the effect of wounding on the organogenic potential of internodal stem explants. Root regeneration was severely affected when a thin cell layer was removed from an internodal stem explant (Table 7.3, Section 7.3.1). Shoot regeneration was also affected, but to a lesser degree, and the extent of reduction was seemingly influenced

by the species and cultivar investigated. The results imply that the presence of an epidermal layer is essential for the plant's rhizogenic potential to be realised. This contrasts with observations made by Chylah (1974) where stem segments of Torenia fournieri Lind were found to be capable of rhizogenesis, only when the epidermis was removed. Furthermore, as histological sections through intact stem segments show, the tissue involved in root formation is of endogenous perivascular origin, therefore it seems surprising that removal of a thin cell layer would affect formation to such a degree. It does suggest however, the existence of inter-tissue correlations which once disrupted, leads to changes in cellular states, which in turn result in different morphogenetic expression. These correlations have been investigated by several workers and their results have demonstrated constraints which tissues within an organ can exert on each other, with respect to morphogenetic expression (Chylah, 1974; Tran Thanh Van, 1981; Walker, 1983). As well as these inter-tissue correlations, responses induced by wounding per se have also to be considered. These include DNA amplification, changes in peroxidase activity, in isozymic pattern and in metabolism of phenolic compounds (Tran Thanh Van, 1981).

A more detailed investigation considered the effect of wounding on the extent and position of the regenerating shoots on the internodal stem tissue. With the diploid species investigated, all adventitious shoots regenerated from the apical end of the explant, and removal of the thin cell layer, or removal followed by replacement of the thin cell layer did not affect shoot regener-

ation. The tetraploid species, on the other hand, was seen to regenerate shoots from both ends of the explant. In this case, removal of the thin cell layer led to an increase in shoot production at the apical end of the explant, whereas, removal followed by replacement resulted in reduced shoot regeneration at both the apical and basal ends of the explant (Table 7.4, Section 7.3.2). Takeuchi et al., (1985) investigated the effect of wounding on adventitious bud formation in cultured stem segments of Torenia fournieri Lind and found adventitious shoot regeneration was favoured by the application of cytokinin through the medium and further wounding of the explants. Most of the adventitious buds were formed at the margins of the explants, and more were formed close to the apical cut ends than at the basal ends, as found with S. sparsipilum in this study. Inflicting further wounds to the tissue in the form of transverse or longitudinal cuts resulted in a significant increase in the number of shoots formed in close proximity to the wounded area; when a wound treatment was given longitudinally to an explant, the number of buds increased over the entire surface of the explant. It was proposed by Takeuchi et al.(1985) that the wounding had affected the uptake into the explants and/or the distribution pattern of the cytokinin (BAP) in the explant, however, radioactive studies did not support the proposal. As reported in Section 7.3.1, removal of a thin cell layer did lead to some adventitious shoot regeneration occurring close to the wounded area, but the majority of regenerants were confined to the cut ends of the explants, in both wounded and unwounded tissues.

The production of shoots only at the apical end of the explant, as observed with explants derived from S. sparsipilum, is possibly a reflection of the basipetal movement of endogenous auxin, resulting in an area of tissue at the apical end of the explant which is low in auxin and relatively high in cytokinin, as a result of absorption from the medium. This high cytokinin : low auxin ratio could therefore be sufficient a stimulus to induce shoot regeneration at the apical end of the explant. However, one must also consider changes occurring at the ends of the explants in response to wounding; these factors are also likely to affect the organogenic potential of the explant in some way. With S. tuberosum ssp tuberosum cv. Pentland Ivory, shoots regenerated from both the apical and basal ends of the explants, with the greater percentage of shoots developing from the basal end. This contrast in the location of the site of regeneration is likely to be the result of several interacting factors: differences in the levels of endogenous growth regulators; differences in wounding response and a differing ability in the rate at which each end of the explant of both species can absorb cytokinin from the medium. Where the latter is concerned there is evidence that synthetic cytokinins are translocated acropetally in plant tissue (Wareing et al., 1981). It is possible that the basipetal end of the explant of the tetraploid species is more efficient than that of the diploid species in absorbing cytokinin from the medium. Alternatively the apical regeneration site of S. sparsipilum could be a reflection of a more effective translocation of synthetic cytokinin than that afforded by cv. Pentland Ivory.

As previously stated the effect of thin cell layer removal on shoot regeneration varied with the two species: no effect was observed with S. sparsipilum whereas the extent of shoot regeneration at the apical and basal ends of the explant was affected with S. tuberosum ssp. tuberosum cv. Pentland Ivory, especially when the thin cell layer was replaced (Section 7.3.2). There is evidence that suggests epidermal layers contain relatively high levels of peroxidase (Andreae et al., 1960). Removal followed by replacement of an epidermal layer to an isolated tissue could result in increased levels of peroxidase within the system. The outcome of this could be a modification of the balance in shoot regeneration potential between the two polar ends of the explant, as a result of the effect of peroxidase on auxin. Nevertheless, this argument is not supported by the results obtained in Section 7.3.1 in which thin cell layer removal resulted in markedly reduced root regeneration. On the basis of the argument above, root regeneration should have been only mildly reduced, if at all, by removal of a thin cell layer. It therefore seems that these apparent connections between rhizogenic potential, caulogenic potential and site of origin and the epidermal (thin cell) layer are the result of several factors activated both by wounding the explant and disrupting inter-tissue correlations, working either in isolation or in combination.

Histological studies of the cultured explant tissues showed that cell divisions initially took place in the vascular area of the explant (Section 7.4). Further culture revealed that this activity had extended to the cortical regions of the explant and early indications of shoot organization were observed near the

explant surface. Although some explants revealed this connecting layer of actively dividing and differentiating tissue close to the explant surface, other explants showed these areas of meristematic tissue to be separated from the original vascular tissue by large, vacuolated cells. These activities preceded the formation of shoot apices with a single, tunica layer (Plate 7.2G, Section 7.6) at the surface of the callus tissue formed at the ends of the explants. Although shoots can arise from peripheral cells without any obvious connections with vascular tissue (Earle et al., 1965; Nuti Ronchi, 1981), it is likely that in those sections where vascular connections were absent, such connections were formed later.

7.14.2. Thin cell layer regeneration

Section 1.4.3 outlines some of the work utilising thin cell layers that has been embarked on over the last 15 years. In the forefront of this work is Tran Thanh Van whose research has shown how thin cell layers of certain plant species can be programmed to express different morphogenetic behaviour depending on the components of the culture medium, and furthermore, have been used to evaluate the concept of inter-tissue correlations. The flexibility of thin cell layers in expressing a particular morphogenetic pathway has not been looked at fully in this investigation, as the main aim was to determine their ability to produce shoots.

Of all the media used (Section 7.5), only three were seen to favour shoot regeneration, and these were the media containing either zeatin or zeatin riboside. The two two-stage procedures,

E31/E41 and E32/E42, which on the whole failed to provide the conditions necessary to induce shoots from either the intact internodal explant (Section 7.2) or the wounded internodal explant (Section 7.3.1) of the two diploid species, proved favourable to shoot formation in the case of the thin cell layers. For example, using the two-stage procedure of E31/E41 (E31 utilises 2,4-D and zeatin and E41 utilises only zeatin) under light conditions of $5\mu Mm^{-2}S^{-1}$ PAR and a 8h daylength, with a temperature of 22 ± 1°C, intact internodal explants of S. chacoense exhibited zero regeneration (Section 7.2), the same result was obtained with wounded explants (Section 7.3.1) whereas with thin cell layers, 42% shoot regeneration took place (Section 7.5). Thus the capacity of thin cell layers of this diploid species to produce shoots was masked in the physiological context of an organ explant and can seemingly only be expressed in the absence of vascular tissues. The same was found with Psophocarpus species (Leguminosae), where thin cell layers were found to be more morphologically competent than the corresponding internode (Tran Thanh Van, 1981).

The medium containing a relatively high concentration of zeatin riboside ($4mgl^{-1}$), which induce maximum shoot regeneration from intact and wounded internodal explants of all species and cultivars, under the majority of light and temperature conditions tested, achieved very little shoot regeneration from the thin cell layers. This could reflect the existence of inhibitory levels of growth regulators, which is possibly connected to the reduced volume of explants, and the presumably lower levels of endogenous growth regulators.

Light was seen to exert an influence on the organogenic response of these thin cell layer explants. As daylength and light intensity increased, organogenic response of the thin cell layers decreased (Section 7.5). This contrasts with the light effect observed during the culture of intact, internodal explants, where increasing daylength and light intensity resulted in increased regeneration. The only reference to the influence of light in the morphogenic development of thin cell layers found in the literature was made by Tran Thanh Van (1973) where results showed how light or darkness could have a determining effect on the type of organ formed. As discussed in Section 7.14.1 light is known to affect morphogenic behaviour in other culture systems and therefore it is understandable that a light factor has been noted in this investigation. The apparent inhibition of organogenesis in thin cell layers by relatively high levels could possibly be connected to the reduced size of the explants. Such a link between size of explant and levels of endogenous growth regulators has been suggested by Tran Thanh Van et al., 1974, and so it is possible that a similar link exists between explant size and light levels, thus leading to the existence of inhibitory light levels.

The results obtained in this investigation and the investigations discussed in Section 7.14.1, do tend to substantiate the idea that within intact systems there are stimulatory and inhibitory correlations, which become evident once that intact system is disrupted. However, as stated in Section 1.4.3, there are limitations to such conclusions which must be accepted until

there is clear understanding as to the effect of wounding; the behaviour of excised thin cell layer cannot strictly be compared with the behaviour of the same cell layer when it is still within the confines of the stem internodal explant.

Histological analysis showed that buds arising from thin cell layer explants are composed of cells derived from both the epidermal and the subepidermal layers. There was no histological evidence to suggest that buds could arise exclusively from epidermal layers (Section 7.6). Chylah (1974), reported on adventitious shoot formation from thin cell layer explants of Torenia fournieri Lind, shoots were seen to arise exclusively from the epidermis or from both the epidermal and subepidermal cells. Tran Thanh Van et al., (1970) noted that shoots regenerating from thin cell layer explants of Nautilocalyx lynchei were derived from epidermal and subepidermal tissue, although there were indications that cell activity began in the epidermal cells.

7.14.3. Regeneration from thin cell layer grafts

As detailed in Section 7.7, the major morphogenic response observed when thin cell layer heterografts were cultured on regeneration media was callus production. The major organogenic response was rhizogenesis with a limited number of explants producing shoots. Thus the type of regeneration had changed completely from when similar tissues were cultured, either as an entire organ (internodal stem section), or the same but lacking a thin cell layer, or as a thin cell layer system. In these systems, shoots were regenerated

seemingly in preference to roots. However, the reverse of this organogenic pattern is seen when similar tissue, but within a grafted system, are cultured on a grafting medium followed by a regeneration medium. As Section 7.3.1 indicated, wounding an internodal explant by removing a thin cell layer markedly inhibits root production, furthermore, culture of thin cell layers on regeneration medium does not favour root production (Section 7.5) suggesting therefore some other explanation as to why root production is the preferential organogenic path expressed when thin cell layer heterografts are cultured. The possibility of a different balance of endogenous growth regulators in the explants arises; that is, has the endogenous auxin content increased, possibly accounting for the increased rooting response. As described in Section 7.9, varying concentrations of different growth regulators were used to culture intact stem internodal explants in an attempt to determine the influence of such growth regulators on the eventual morphogenetic competence of stem explants. Results showed that the regeneration potential of both diploid and tetraploid species were affected, with the diploid species being more markedly affected, for example, with S. sparsipilum shoot regeneration on medium containing zeatin riboside ($4mgl^{-1}$) under a light intensity of $85\mu Mm^{-2}s^{-1}$ PAR (16h daylength) and at a temperature of $22 \pm 1^{o}C$ was 100% without the period of culture on a grafting medium (2.0NAA/2.0BAP)(Section 7.2) and 0% when this period was included (Section 7.9). This inhibitory influence on regeneration was less evident when NAA and BAP in the grafting medium were substituted with other growth regulators, but these combinations of

growth regulators had failed in previous experiments to achieve successful graft unions. Thus it would appear that there is a carry-over of growth regulators from the grafting medium in the cultured tissues which affects the future regeneration potential of that explant, with tetraploid tissue being affected to a lesser degree than diploid tissue.

This idea was further examined through the use of a period in between the grafting and regeneration stage during which explants were cultured on a media containing no growth regulators with or without activated charcoal. Once again this experiment showed the inhibitory effect culture on the grafting medium (2.0NAA/2.0BAP) has on the regeneration potential of the diploid species (Section 7.10). Culture on a medium containing activated charcoal prior to culture on a regeneration medium, and after culture on a grafting medium, improved shoot and root regeneration in thin cell layer heterografts of the diploid donor tissue/tetraploid recipient tissue type, but had no effect on heterografts of the reverse order, or internodal stem explants. Activated charcoal has been used by several workers in tissue culture systems, for example, Kohlenbach et al., 1978; Weatherhead et al., 1979; Wenzel et al., 1983. Its effects are recognised as resulting from its ability to adsorb many types of molecules, however, its precise function and action are largely unknown. Therefore, it is difficult to be certain why its presence improved regeneration in some of the explants and not in others. Presumably heterografts composed of diploid donor tissue and tetraploid recipient tissue (2n→4n) presented a system whereby whatever was leading to reduced regener-

ation could be removed or modified by using activated charcoal in the medium.

Shoot and root formation from the thin cell layer heterografts achieved maximum levels when the regeneration medium contained $4mgl^{-1}$ zeatin riboside (4ZR)(Section 7.7). The number of explants producing shoots was small but multiple shoot formation occurred from this small number, especially when the length of culture was four to six months. Shoot production was favoured by grafts composed of diploid donor tissue and tetraploid recipient tissue, presumably because this presented a system in which the larger portion of the graft was tissue seemingly unaffected by the growth regulators in the grafting medium, that is, tetraploid tissue (see Section 7.9). In addition, such a graft combination would mean that the tetraploid tissue, with its apparent lack of sensitivity to exogenous growth regulators compared to diploid tissue, was in direct contact with the medium and therefore likely to be acting as a type of barrier or filter to the growth regulators entering the grafted system and eventually the diploid donor layer from the grafting medium. Furthermore, as shown in Section 7.5 the thin cell layers of the diploid species do appear to be more organogenically competent than those of the tetraploid species, and thus as the donor component of the graft, would be more likely to allow shoot regeneration to occur than if the tetraploid tissue occupied the donor position.

The same limits exist when making comparisons between intact, non-grafted internodal explants and grafted internodal explants,

as do exist between an excised thin cell layer and a thin cell layer in situ on an internodal stem explant. A heterograft achieved through combining diploid and tetraploid tissue cannot be expected to behave as either tissue would in isolation; or, alternatively as a combination whose response in culture can be predicted on the basis of responses observed during culture of individual components. In addition to the level of endogenous growth regulators, already discussed, other factors are likely to affect regeneration potential. The ability to form polarised gradients of phytohormones could be affected by grafting tissue of different ploidy levels together. Sinnott (1960) has suggested that polarity is the first requirement for organized development in plants. With this in mind, some form of polarity is more likely to be established between two tissues whose cell size and number of cells are similar, in that they have been derived from tissue of the same ploidy level rather than two tissues which have originated from two different species. There is also the question of mutual cellular interaction. If such interaction is based on genotype-specific morphogens, then there is the possibility that morphogens capable of inducing regeneration in one species, will not be able to induce a similar response in another species.

Sections of thin cell layer heterografts were taken and examined as described in Section 7.11. Analysis of these sections gave no indication of the cellular involvement which occurs when shoots arise from such explants. On the other hand, root initiation was always seen as emanating from within the original vascular region of the recipient tissue, or in very close proximity

to it. Potential shoot buds, however, were observed as originating from the callus tissue which had developed from the recipient tissue (Plate 7.7C, Section 7.11) from what seemed to be areas of exclusively donor tissue (Plate 7.7B, Section 7.11) and also from areas which were so positioned to suggest that the tissue could be of mixed genotype (Plate 7.7G, Section 7.11). This suggests that induction of these adventitious shoots depends to a large degree on localised levels of morphogens and that these levels in such a complex system as a thin cell layer heterograft would develop to a large degree by chance, and would vary with individual explants. This concept of shoot formation occurring as the result of a balance of various substances, indluding growth regulators and metabolites, at particular loci, is proposed by several workers as the means by which shoots develop from disorganized tissue such as callus (Ross et al., 1973; Nuti Ronchi, 1980).

The organogenic response of thin cell layer autografts to several regeneration media was described in Section 7.8. These results showed that the organogenic competence of the autografts is less affected by the grafting process than that of the heterografts. Shoot and root production are reduced but to a lesser degree than that noted when the grafts were composed of tissue of different ploidy. Bearing in mind previous deductions made as to the effect on the diploid species when cultured on grafting medium, prior to culture on regeneration medium (Section 7.9) one would expect an autograft comprising of diploid tissue to show little organogenic ability, however, as Table 7.6 (Section 7.8) shows, given the conditions found to encourage maximum regeneration in intact

stem internodal explants, 50% shoot formation was achieved from autografts consisting of diploid tissue. This therefore implies that the marked reduction in regeneration potential which occurred with the culture of heterografts was mainly the result of culturing tissues of different ploidy levels together. Furthermore, whatever was responsible for inhibiting regeneration in intact stem internodal explants of the diploid species (S. sparsipilum) after they had been cultured on the grafting medium (Section 7.9), was apparently negated by the actual grafting process. With respect to the latter, it is likely that the grafting process, that is, wounding and graft development, would modify the balance of growth regulators and metabolites to such an extent that the endogenous make-up of a diploid autograft would be very different from that of an intact diploid stem internodal explant, even though they had been cultured on the same grafting medium. The positive response of the autografts of both species to the regeneration medium compared with the reticence exhibited by the heterografts could also be a further indication of the inability of the two tissues to form polarised gradients of phytohormones.

7.14.4. Regeneration from callus

Regeneration from callus, generally, was poor, whether or not the callus originated from one species or two species of different genotype (mixed callus). As reported in Section 7.12, the major organogenic response was that of root formation, with a very limited number of explants, from one species only, producing shoots.

This tendency towards root formation does occur more frequently in callus cultures than shoot formation, and it has been suggested that this is related to an altered morphogenetic potential (Negrutiu, 1978). The ability of callus to express any organogenic competence can be affected by a number of factors which have already been discussed in Section 1.4.1. Two factors which have been noted as interfering with organogenesis are, the use of 2,4-D in the culture medium, and length of time over which the callus is cultured. With respect to the former, this does not appear to be of significance in inhibiting regeneration from the callus investigated in these experiments, for as reported in Section 7.13, only those callus explants induced and maintained on 2,4-D were found to be eventually capable of shoot and root regeneration. There have been reports in the literature concerning the connection between chlorophyll formation and morphogenetic potential, and that auxin can inhibit the former, and therefore indirectly affect the latter (Hildebrandt et al., 1963; Van Huystee, 1977). In this investigation, however, the ability to synthesise chlorophyll seemed to be associated with the individual explant rather than cultural conditions, and was not necessarily an indication of eventual organogenic expression. As indicated by the results reported in Section 7.13, age of callus, that is, duration of culture appeared to have no effect on the establishment of an organogenically competent callus system, with the use of a different regeneration medium.

Culture of the callus using a two-stage procedure (D1 and D2) resulted in maximum shoot and root regeneration from callus of single species and mixed species origin, with the regenerative ability of the former exceeding that of the latter. Of the single species calluses, callus derived from the tetraploid species showed better ability at producing shoots and roots than callus derived from diploid species (Table 7.12, Section 7.13). These media are fairly complex in their composition, but one factor of interest is the change in the sucrose concentration to which the callus is exposed through culture on these media (from 4.0% (w/v) on callus maintenance medium to 0.25% (w/v) on D1 medium to 2.5% (w/v) on D2 medium). As reported in Section 1.4.1, there have been reports that prolonged culture of cells on sucrose can inhibit shoot regeneration (Van Huystee, 1977; Rains et al., 1980). Shepard (1982) also presents results which point to the sensitivity of morphogenetic processes in potato to the exogenous supply of sucrose. The relative success of these media (D1 and D2) provide further evidence for the stimulatory effect of zeatin on adventitious shoot formation in potato, as zeatin is present in D2 medium. Furthermore, D2 medium contained abscisic acid which Shepard (1980) reported as a beneficial addition to regeneration medium for cultured tuber tissues and protoplast-derived callus of potato.

Callus derived from tissues such as stems is generally a complex mixture of differentiated cell types with varying sensitivities to imposed stimuli. Therefore a relatively complex medium (such as D1 and D2) can stimulate a wide variety of cells

with differing proliferative capacity, ploidy level and physiology within the population. In addition, as discussed by Torrey (1965), continued subculture of callus may select cell types eliminating certain cell populations and perpetuating others. Thus, within a callus explant derived from a single species, there are likely to be sufficient complexities to impede the organized development necessary for shoot formation. A mixed callus system would presumably add to these complexities in that the original source of mixed callus was derived from two tissues of different ploidy levels. Thus the co-ordination necessary within such a system so that a precise balance of regeneration-inducing substances arrive at a particular point in the cultured tissue where the cells are competent to react is likely to be more complicated and possibly explains the limited organogenic potential expressed by the mixed species callus.

CHAPTER 8

IDENTIFICATION OF REGENERANTS

8.1. INTRODUCTION

Identification of chimeral shoots can present problems. Ideally a chimeral system should be developed whereby some visual character can be used to identify the presence of chimeral shoots (Marcotrigiano et al., 1984). However, in the absence of this direct form of identification, cytological analysis can be used. In this study, chromosome counts of root-tip tissue have been used to identify the L2/L3 layers (Sree Ramulu et al., 1975; Tilney-Bassett, 1986), and the guard cell length of the stomata has been the method used to determine the genetic nature of the L1 layer (Satina et al., 1940; Baker, 1943; Horak, 1972). Morphological differences, such as leaf shape and colour, plant growth habit, have also been examined. However, the contribution of this information to the identification of the cell layers within the plant, has been considered as merely corrabatoratory, as workers have shown that such evidence, if given too much importance, can be misleading (Arisumi, 1964).

8.2. STOMATAL GUARD CELL LENGTH OF PLANTS CULTURED IN VITRO

All shoots regenerated from a potential chimeral system were subjected to this analysis (Sections 7.7 and 7.13). On regeneration, shoot-tips were isolated and transferred to semi-solid basal culture medium (Section 2.2) supplemented with 4% sucrose (w/v). The second and third leaves of these shoot-tip derived plantlets (in culture for six weeks) were used in stomatal

guard cell length determination, as described in Section 2.7; from each of these two leaves, the lengths of ten stomatal guard cells scattered throughout the leaf were measured. In order to determine the effect that the growth regulators used in grafting and regeneration procedures might have on stomatal development in in vitro plantlets, the stomatal guard cell-lengths from the following sources were also measured: plantlets regenerated from successful autografts (Section 6.2.13) of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory after culture on 4ZR medium (Section 7.8); plantlets derived from shoots isolated from callus explants of S. sparsipilum and cv. Pentland Ivory, in which the callus had been induced and maintained on 2,4-D medium for four months (Section 4.2) and shoot regeneration had been initiated on D1/D2 media (Section 7.13). Similar determinations were carried out on shoot-tip derived plantlets, of the same species as previously stated, maintained as stock material on basal culture medium (Section 2.2) supplemented with 4% sucrose (w/v): for each species the number of plantlets investigated was 20, and the number of stomata on each plantlet examined was ten (five each from the second and third leaves as described earlier).

Measurements of the stomatal guard cell lengths of the plantlets derived from the stock material indicated mean values of 30.00 ± 1.36μ for the diploid species and 38.73 ± 1.29μ for the tetraploid species. Calculation of the 99% confidence interval for both means resulted in 30.00 ± 0.87 as the range determining those plantlets with a diploid L1 layer, whereas those which fell within the range of 38.73 ± 0.83 were considered to have a tetra-

ploid L1 layer (see Appendix II.4). These two populations were compared using a student's t-test which indicated that the stomatal guard cell length mean for the diploid population was significantly different from that of the tetraploid population.

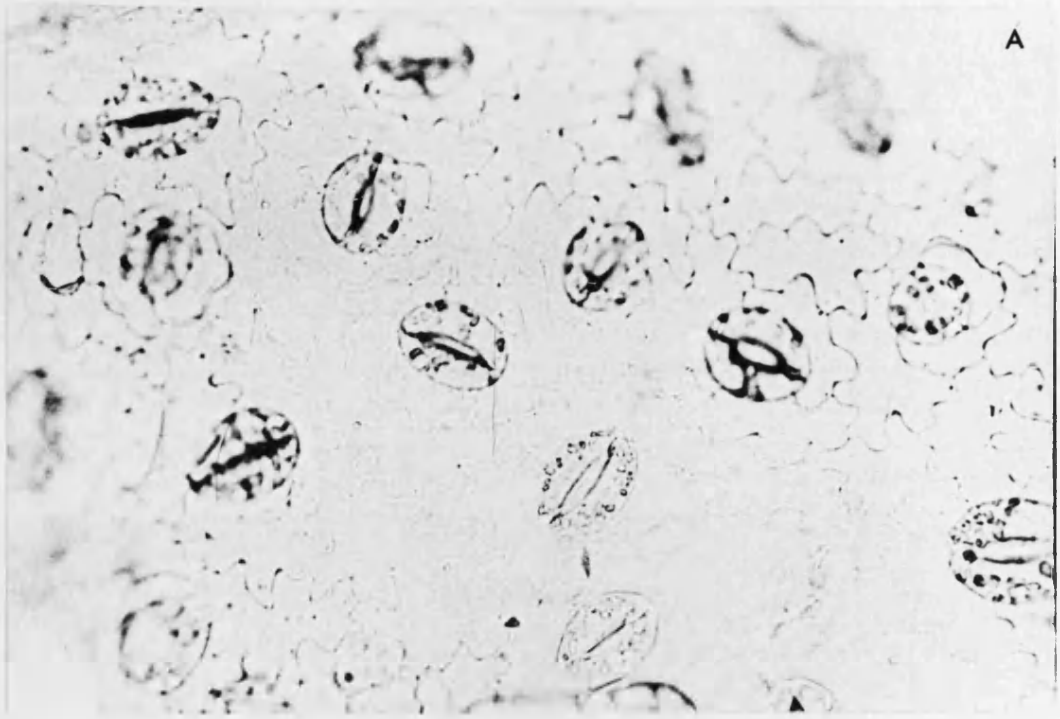
The stomatal guard cell lengths of the other control plantlets previously described were found to lie within the limits outlined by the 99% confidence intervals, that is, 30.00 ± 0.87μ if diploid and 38.73 ± 0.83μ if tetraploid (Table 8.1). Of the 255 plantlets which had regenerated from either the grafting system or the mixed callus system, six were found to have stomatal guard cell lengths indicative of a diploid L1 layer, according to the 99% confidence interval. All other plantlets examined were found to have stomatal guard cell lengths outside this range with the exception of two regenerants originating from a heterograft composed of tetraploid donor tissue and diploid recipient tissue. Chromosomal analysis (Section 8.2) later revealed the L3 layer of these plantlets also to be diploid.

Plates 8.1A and 8.1B show to the same scale, stomata representative in size and density of the diploid S. sparsipilum and the tetraploid S. tuberosum ssp. tuberosum cv. Pentland Ivory. Table 8.1 shows the stomatal guard cell lengths from a selection of the 261 plantlets examined. The selection includes the control plants and the six plantlets previously outlined.

Plate 8.1. The stomata of shoot-tip derived plantlets (cultured for six weeks) of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory

A. The stomata of S. sparsipilum. Diploid species examined in this study were found to have a stomatal guard cell length of 30.00 ± 1.36μ x 1093

B. The stomata of S. tuberosum ssp. tuberosum. Tetraploid species examined in this study were found to have a stomatal guard cell length of 38.73 ± 1.72μ x 1093


A


B

8.3. CHROMOSOME COUNTS OF PLANTS CULTURED IN VITRO

Feulgen-stained squash preparations were made from rapidly growing root-tips developing from the shoot-tips isolated from potential chimeral systems. Root growth was induced as a result of culture on semi-solid basal medium (Section 2.2) supplemented with 4% sucrose (w/v). Counts were taken from a minimum of five well-spread cells and at least two separate roots, and chromosomes were prepared as described in Section 2.8.2. Preparations of root-tips were made from all the regenerants isolated from potential chimeral systems and from the plantlets described in Section 8.1.

Cytological preparations of root-tips from S. sparsipilum consistently showed the diploid number of 24 chromosomes (Plate 8.2A). The chromosome number of S. tuberosum ssp. tuberosum cv. Pentland Ivory was confirmed as $2n = 4x = 48$ (Plate 8.2B). There was no deviation from this number on examination of chromosomes in root-tips of plantlets induced to regenerate from autografts or callus tissue through the use of growth regulators in the medium; this also applied to tissue derived from S. sparsipilum. Table 8.1 shows the chromosome numbers of some of the plantlets investigated. Section 8.1 outlines which plants were chosen to be included in the table. In addition, the small number of aneuploids (5%) which were detected (Plate 8.2C) with chromosome numbers ranging between 46 and 48 (47 being the most frequent) were also included in Table 8.1.

TABLE 8.1. STOMATAL GUARD CELL LENGTH AND CHROMOSOME NUMBER ANALYSIS

ORIGIN OF REGENERATED SHOOT	MEAN STOMATAL LENGTH (μ)	CHROMOSOME NUMBER (FROM ROOT-TIP CELLS)
S. sparsipilum	30.18	24
S. tuberosum ssp. *tuberosum* cv. Pentland Ivory	38.56	48
Callus derived from stem tissue of *S. sparsipilum*	30.26	24
Callus derived from stem tissue of cv. Pentland Ivory	39.56	48
Autografts of *S. sparsipilum*	29.89	24
Autografts of *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory	39.24	48
(1) HG 2n → 4n	30.62	48
(2) HG 2n → 4n	29.37	48
(3) HG 2n → 4n	29.42	48
(4) HG 2n → 4n	30.17	48
(5) HG 2n → 4n	29.67	48
(6) HG 2n → 4n	29.62	48
HG 2n → 4n	33.71	46
HG 2n → 4n	37.67	47
HG 2n → 4n	37.83	47
Mixed callus	45.24	46
Mixed callus	40.26	47
Mixed callus	43.29	47

TABLE 8.1. CONTINUED

ORIGIN OF REGENERATED SHOOT	MEAN STOMATAL LENGTH (μ)	CHROMOSOME NUMBER (FROM ROOT-TIP CELLS)
Mixed callus	37.71	47
HG 2n → 4n	42.86	47
Mixed callus	36.00	46
Mixed callus	34.25	47
Mixed callus	33.87	47
Mixed callus	34.50	47

Key:

HG : heterograft

2n → 4n : diploid donor tissue (S. sparsipilum) grafted onto tetraploid (S. tuberosum ssp. tuberosum cv. Pentland Ivory) recipient tissue

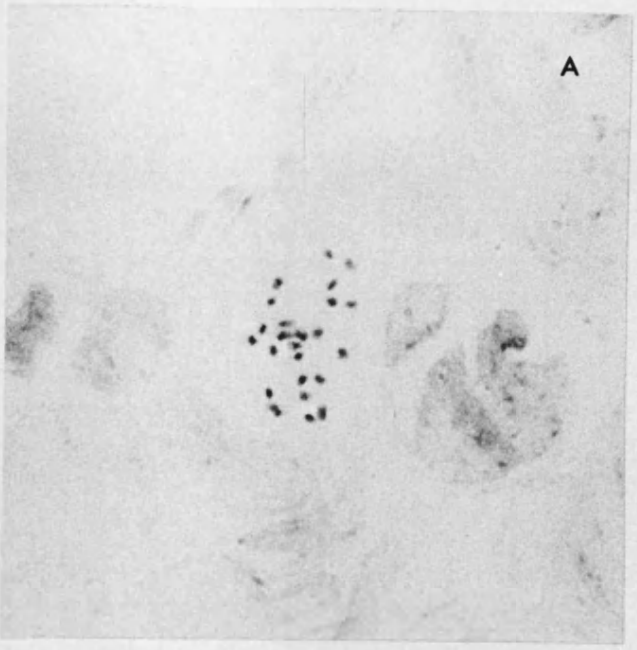
Numbers one to six : six plantlets selected as possible chimeras on the basis of stomatal and chromosome analyses

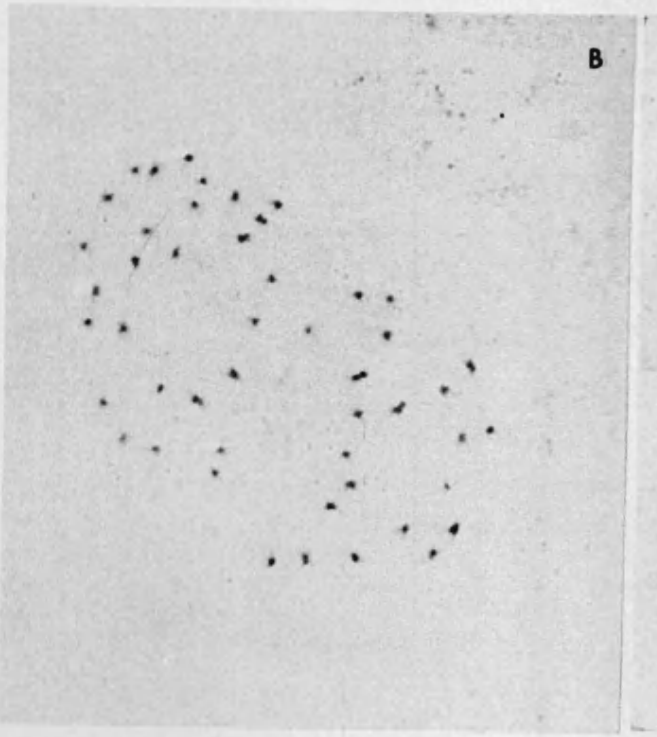
Plate 8.2. Chromosome numbers in root-tips of plantlets derived in vitro

A. S. sparsipilum (2n = 2X = 24)

B. S. tuberosum ssp. tuberosum cv. Pentland Ivory (2n = 4X = 48)

C. Regenerant from thin cell layer heterograft where S. sparsipilum was the donor component and S. tuberosum ssp. tuberosum cv. Pentland Ivory was the recipient component of the heterograft (2n = 4X =47)


A


B


C

8.4. ADVENTITIOUS SHOOT FORMATION FROM CULTURED POTATO ROOTS

Consideration was given to the possibility of inducing shoot regeneration from the root tissue of the plantlets derived from potential chimeral systems, as these shoots would be representative of the L3 layer (Howard 1964a; Miedema, 1967). Subsequent cytological analysis of the regenerating shoots could provide further evidence as to the genetic nature of the plantlet. Espinoza et al., (1985) isolated roots from plantlets maintained in vitro of five different genotypes and succeeded in inducing shoot regeneration from four of the five genotypes investigated. The majority of shoots regenerated (80%), with no apparent callus phase. Consequently, the method used by Espinoza et al., (1985) was utilised in this experiment to obtain shoot regeneration from cultured root tissue.

Plantlets of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory were removed from culture dishes and the roots were cut into 3cm long segments to be inoculated into petri dishes containing two different experimental media. Basal culture medium (Section 2.2) supplemented with BAP ($1mgl^{-1}$), IAA ($1mgl^{-1}$) and GA_3 ($10mgl^{-1}$) was used. Sucrose was added at two different concentrations: 2.5% and 5.0% (w/v). Once inoculated, the roots were cultured at 22 ± 1°C; two light intensities of 16h daylength were employed: 20 and $85\mu Mm^{-2}s^{-1}$PAR. For each medium and light intensity combination, 30 to 40 explants were cultured.

After 16 days of culture, roots from both species had produced greenish-yellow callus of a moist, soft appearance. With S. sparsipilum, callus was produced at points along the length of the isolated roots, whereas with cv. Pentland Ivory, callus production occurred, as a general rule, at the ends of the isolated roots and gradually extended inwards (Plate 8.3A). With this genotype, 5% sucrose, at the lower light intensity, appeared to favour callus production, whereas at the higher light intensity, 5% sucrose appeared to inhibit callus production. With S. sparsipilum, the higher sucrose concentration favoured callus production at both light intensities.

After 50 days of culture, root regeneration had occurred from the root tissue of S. sparsipilum, but only from those roots cultured under 85$\mu Mm^{-2}s^{-1}$PAR. Both sucrose concentrations gave similar levels of root regeneration: 3% (2.5% sucrose) and 5% (5.0% sucrose). Final assessment was made after 113 days of culture (see Table 8.2). At this stage, there had been no change in the morphogenetic response of S. sparsipilum, in addition, the green callus was being replaced by brownish-yellow callus. The callus from cv. Pentland Ivory, however, remained greenish-yellow in colour, and under both light intensities, and with both sucrose concentrations, had produced shoots. Shoot production was favoured at the lower sucrose concentration and under the higher light intensity (Table 8.2). However, the shoots which regenerated, were shiny and translucent in appearance (Plate 8.3B) and basically looked abnormal. As a result, and also because of the shortage of time, this experiment was not taken any further.

TABLE 8.2. Adventitious shoot formation from cultured potato roots

SPECIES/ CULTIVAR	LIGHT INTENSITY $\mu Mm^{-2}s^{-1}$ PAR	SUCROSE CONCENTRATION (%)	% EXPLANTS PRODUCING SHOOTS	% EXPLANTS PRODUCING ROOTS
S. tuberosum ssp. *tuberosum*	25	2.5	2.5	2.5
cv. Pentland Ivory	25	5.0	5.0	3.0
cv. Pentland Ivory	85	2.5	15.0	-
cv. Pentland Ivory	85	5.0	8.0	-
S. sparsipilum	25	2.5	-	-
S. sparsipilum	25	5.0	-	-
S. sparsipilum	85	2.5	-	3.0
S. sparsipilum	85	5.0	-	5.0

Key:

Media: MS + BAP ($1mgl^{-1}$), IAA ($1mgl^{-1}$) GA_3 ($10mgl^{-1}$) + sucrose (2.5% or 5.0%)

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 20 or $85\mu Mm^{-2}s^{-1}$ PAR

Number of replicates per treatment: 30 to 40

Plate 8.3. Adventitious shoot formation from roots of *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory cultured on a medium containing BAP ($1mgl^{-1}$), IAA ($1mgl^{-1}$), GA_3 ($10mgl^{-1}$) and sucrose (2.5% w/v)

A. Callus which developed on roots excised from cv. Pentland Ivory was confined to the ends of the isolated roots. Callus was present after 16 days of culture x 6

B. Plate 8.3A in more detail showing shoot regeneration after 100 days of culture. These shoots were shiny and translucent in appearance and did not look as if they would develop into normal plantlets if removed from the callus tissue and cultured on the appropriate medium x 16


A


B

8.5. MORPHOLOGY OF REGENERANTS

Morphological differences of regenerants were examined, both in the early stages of plantlet development, that is, after three to four weeks of culture on basal culture medium (Section 2.2), and when the plants had been established *in vivo*. Only those plantlets indicated as potential periclinal chimeras by stomatal and chromosomal analysis, were examined morphologically. Of the 255 plantlets examined, only six fulfilled these requirements, and were studied morphologically. These six potential periclinal chimeras (numbered one to six in Table 8.1) originated from two thin cell layer heterografts in which the donor tissue was diploid (*S. sparsipilum*) and the recipient tissue was tetraploid (*S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory).

8.5.1. *In vitro* morphological examination

Plantlets produced as described above were studied after transfer to basal culture medium (Section 2.2) supplemented with 4% sucrose (w/v). Comparisons were made between these plantlets and plantlets which were derived from autografts of *S. sparsipilum* and cv. Pentland Ivory; as products of regenerative autografts, they had been subjected to the same growth regulators as the potential chimeral plantlets. In addition, the morphology of shoot-tip derived material of *S. sparsipilum* and cv. Pentland Ivory maintained as stock material, was examined. Table 8.3 outlines the morphology of the plantlets examined.

Key:

Hair density: 1 = short, fine hairs visible under microscope

2 = similar to 1, but greater density

3 = thick covering of long hairs

HG: heterograft

2n → 4n: Diploid donor tissue grafted onto tetraploid recipient tissue

Numbers one to six: Six plantlets selected as possible chimeras on the basis of stomatal and chromosomal analyses

TABLE 8.3. Morphology of shoot-tip derived, autograft-derived and heterograft-derived plantlets, in vitro

PLANTLET NUMBER AND ORIGIN	ANTHOCYANIN CONTENT	INTER-NODE LENGTH	LEAF MORPHOLOGY		
			COLOUR	HAIR DENSITY	SHAPE
S. sparsipilum	present in stem	12mm	Dark green	2	Imparipinnate compound leaf with acute apex to terminal leaflet
cv. Pentland Ivory	absent from stem	6-7mm	Green	<1	Simple leaf with rounded apex
S. sparsipilum (autograft-derived)	present in stem	10mm	Dark green	3	Imparipinnate compound leaf with acute apex to terminal leaflet
cv. Pentland Ivory (autograft-derived)	absent from stem	6mm	Green	1	Simple, ovate leaf with rounded apex
(1) HG 2n → 4n	present in stem	5mm	Dark green	>3	Simple, ovate leaf with rounded apex
(2) HG 2n → 4n	absent from stem	6mm	Dark green	>3	Simple, ovate leaf with rounded apex and curled, twisted margin
(3) HG 2n → 4n	absent from stem	6mm	Dark green	>3	Simple, ovate leaf with acute apex
(4) HG 2n → 4n	present in lower surface of leaves	5mm	Dark green	>3	Simple, ovate leaf with acute apex
(5) HG 2n → 4n	present in stem	7mm	Dark green	>3	Simple, ovate leaf with rounded apex
(6) HG 2n → 4n	absent from stem	8mm	Pale green	>3	Simple, ovate leaf with rounded apex. Wrinkled, curled and twisted margin

8.5.2. *In vivo* morphological examination

Shoot-tip derived plantlets of *S. sparsipilum* and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory together with the heterograft derived plantlets, numbers one to six, were established in soil as detailed in Section 2.5. Growth and development of these plants was monitored 14, 21, 30 and 50 days after transfer to soil. 36 days after transfer to soil, *S. sparsipilum* and cv. Pentland Ivory were repotted into larger pots. Table 8.4 describes certain morphological characteristics of these plants 50 days after transfer to soil. Plates 8.6A to 8.6C show the growth and development of *S. sparsipilum*, *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory (Plate 8.6A); plants numbers one and three (Plate 8.6B) and plants numbers two, four and five (Plate 8.6C). Plant number six did not survive *in vivo*. Plate 8.6D shows the shape and size of leaves isolated from *S. sparsipilum*, cv. Pentland Ivory, and plants numbers one, five and two. The leaves chosen for comparison were the third leaves from plants which had been grown *in vivo* for 50 days.

TABLE 8.4. Morphology of shoot-tip derived and heterograft-derived plants *in vivo*

PLANTLET NUMBER AND ORIGIN	NUMBER AND HEIGHT (mm) OF LEAFY SHOOTS	STEM MORPHOLOGY	TUBER FORMATION	LEAF SIZE (WIDTH IN mm)	LEAF SHAPE
S. sparsipilum	3: 175; 250; 210	Red/green and hairy	-	24; 32; 27	Imparipinnate with small leaflets intervening between the pinnae. Rounded apex
cv. Pentland Ivory	4: 134; 245; 25; 10	Green	-	37; 31; 15; 10	As *S. sparsipilum* but with acute apex
1. HG 2n → 4n	4: 60; 60; 60; 45	Red and hairy	Vast tuberisation occurring at base of plant and very small tubers developing from axillary buds	24; 23; 20; 24	Similar to cv. Pentland Ivory in shape but unifoliate i.e. not dissected
2. HG 2n → 4n	2: 70; 8	Red/green and hairy	Very small tubers forming at base of plant	40; 40	As number 1 but showing irregularities in leaflet margin. Possible formation of leaflets at base of terminal leaflet but seemingly fused

TABLE 8.4. (CONTINUED)

PLANTLET NUMBER AND ORIGIN	NUMBER AND HEIGHT (mm) OF LEAFY SHOOTS	STEM MORPHOLOGY	TUBER FORMATION	LEAF SIZE (WIDTH IN mm)	LEAF SHAPE
3. HG 2n → 4n	2: 60; 60	Green and hairy	As number 2	26; 26	As number 1
4. HG 2n → 4n	2: 53; 8	Green and hairy	-	20; 20	As number 1
5. HG 2n → 4n	4: 105; 50; 40; 30	Red and hairy	-	29; 17; 19; 16	As number 1

Key:

HG: Heterograft

2n → 4n: Diploid donor tissue grafted onto tetraploid recipient tissue

Numbers one to six: Six plantlets selected as possible chimeras due to stomata and chromosome analysis

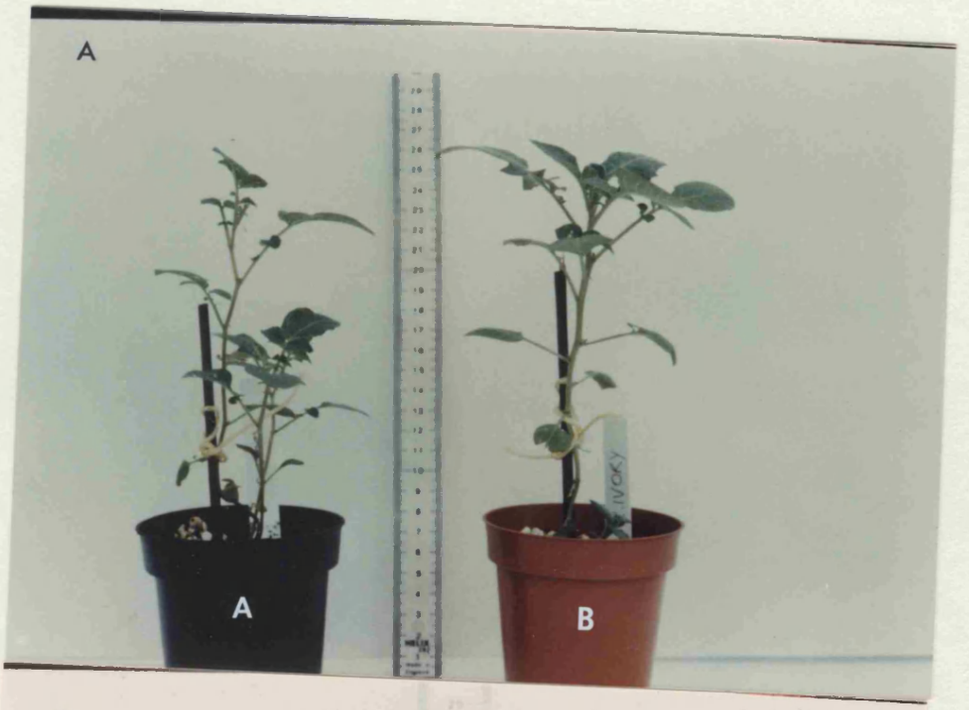
Number six: Plant died 20 days after transfer to soil

Plate 8.6. Morphology of potato plants 50 days after transfer from an *in vitro* environment to soil

A. The growth and development of *S. sparsipilum* (A) and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory (B) These plants originated from shoot-tips of plantlets *in vitro*.

B. The growth and development of two potential periclinal chimeras (numbers three (A) and one (B)). These plants originated from thin cell layer heterografts consisting of *S. sparsipilum* (donor tissue) and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory (recipient tissue)

C. The growth and development of three potential periclinal chimeras (numbers five (A), four (B) and two (C). These plants originated from thin cell layer heterografts consisting of *S. sparsipilum* (donor tissue) and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory (recipient tissue)


A
A
B


B
A
B

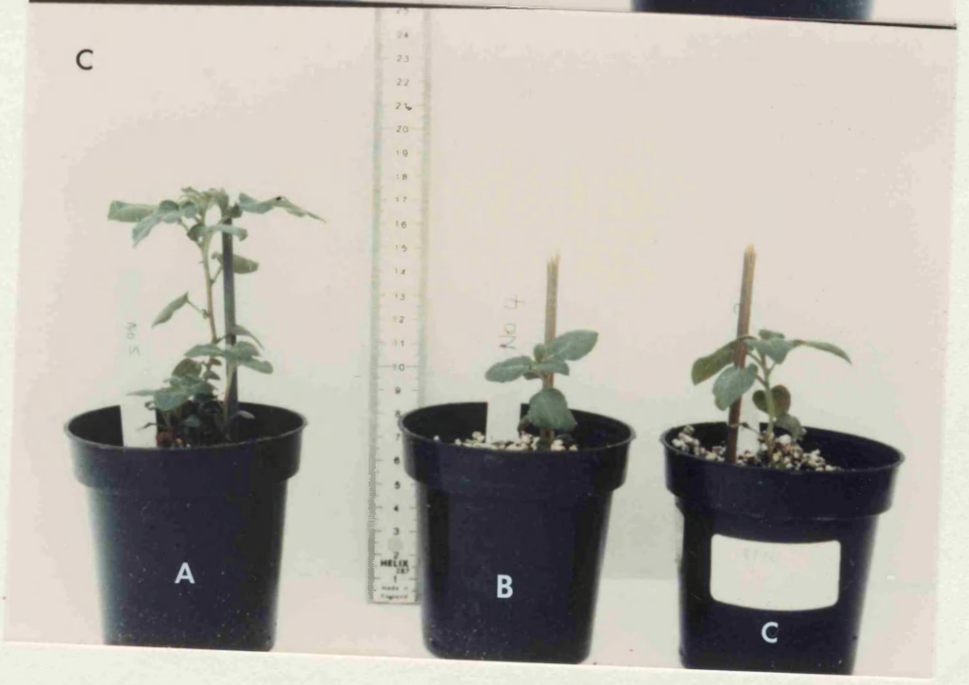

C
A
B
C

Plate 8.6. Leaf shape of potato plants 50 days after transfer from an *in vitro* environment to soil

D. The third leaves of the following potato plants after 50 days growth *in vivo* x 0.6

A: *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory

B: *S. sparsipilum*

C: Potential periclinal chimera number two

D: Potential periclinal chimera number five

E: Potential periclinal chimera number one


D
A
B
C
D
E

8.6. STOMATAL LENGTH AND CHROMOSOME NUMBER ANALYSIS OF PLANTS GROWN IN *IN VIVO* FOR 50 DAYS

Stomatal length measurements and chromosome number analysis were carried out on the plants selected as potential periclinal chimeras, 50 days after their transfer to soil. In addition, plants derived from stock material (Section 2.1) and grown in soil for 50 days were subjected to the same analysis.

8.6.1. Stomatal length measurements of plants grown *in vivo*

The second and third leaves of the plants described above were used for stomatal guard cell length determinations (Section 2.7). From each of these two leaves, the lengths of ten stomatal guard cells scattered throughout the leaf were measured. As Table 8.5 shows the stomatal guard cell length measurements obtained from these plants grown *in vivo* are very similar to those measurements obtained for the same plants when they were in culture *in vitro*. The measurements taken from the control plants, that is, plants derived from stock cultures, indicated mean values of 29.9 ± 0.38μ for the diploid species and 38.6 ± 0.39μ for the tetraploid species.

8.6.2. Chromosome counts of plants cultured *in vivo*

Feulgen-stained squash preparations were made from rapidly growing root-tips developing from the plants described in Section 8.5. Counts were taken from a minimum of five well-spread cells and at least two separate roots, and chromosomes were prepared as described in Section 2.8.2. Table 8.5 shows the chromosome numbers of the

plants subjected to this investigation. These results confirmed the data obtained with *in vitro* plantlets (Section 8.3): the chromosome number of *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory and *S. sparsipilum* was 48 and 24 respectively; the chromosome numbers of the five putative chimeras were as determined in Section 8.3 when the plants were in the *in vitro* stage.

TABLE 8.5. Stomatal length and chromosome number analysis of plants grown *in vivo* for 50 days

ORIGIN OF PLANT	MEAN STOMATAL LENGTH (μ)	CHROMOSOME NUMBER (ROOT-TIP CELLS)
S. sparsipilum (stock)	29.9	24
S. tuberosum ssp. *tuberosum* cv. Pentland Ivory (stock)	38.6	48
(1) HG 2n → 4n	30	48
(2) HG 2n → 4n	29.2	48
(3) HG 2n → 4n	29.5	48
(4) HG 2n → 4n	29.8	48
(5) HG 2n → 4n	29.8	48

Key:

HG: Heterograft

2n → 4n: diploid donor tissue (*S. sparsipilum*) grafted onto tetraploid recipient tissue (*S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory)

Numbers one to five: five of the six plantlets selected as possible chimeras on the basis of chromosome and stomatal analyses

All data for the stomatal length measurements is given in Appendix II.6.

8.7. DISCUSSION

The techniques described in Chapter 8 resulted in the selection of six plantlets whose tissues appeared to be composed of layers of different ploidy. According to the techniques used, each of the six plantlets were made up of a diploid epidermal layer (L1) and a tetraploid L3 layer; the former was determined by measuring the stomatal guard cell length (Section 8.2), and the latter through counting the number of chromosomes in the root-tip (Section 8.3). As discussed in Section 1.2.2 both methods have been used reliably in the past to determine the ploidy levels of the appropriate layers within a chimeral plant. The use of stomatal length in ascertaining the ploidy level of the L1 layer has been confined, as far as is known to *in vivo* plants. Although the results obtained through the stomatal analysis of the potential chimeras after their establishment *in vivo* corroborated those results obtained from stomatal analysis of the same plants *in vitro*, it is worth considering the validity of the stomatal measurements obtained from the *in vitro* plantlets. This is of importance as the six plantlets were selected from the 255 examined on the strength of results obtained through stomatal and chromosomal analysis *in vitro*. Stace (1965) reports that very immature leaves are of no taxonomic use with regard to either macroscopic or microscopic features. However, the same author goes on to say that the epidermis is the first layer to show completion of cell division and that generally stomata reach their maturity before the leaf of the plant attains its mature size. Miedner *et al.*, (1968) point out that there is a tendency for leaves higher up the stem to have smaller stomata and more per unit area than leaves of

lower insertion on the stem. Consideration was given to this when leaves were selected for stomatal measurements, in that leaves in the same position in relation to the apical meristem were chosen from plantlets of the same age. According to Meidner et al., (1968) environmental factors can affect stomatal size and frequency. Environmental factors such as light intensity and duration, and temperature, and to a lesser degree humidity, were in the same order for all the plantlets investigated.

Effects due to different culture procedures such as wounding in the formation of grafts, growth regulators in the media used, were examined in looking at the stomatal size of plantlets other than those thought to be chimeras; for example, regenerants from autografts (Section 8.2). Furthermore, the statistical analysis of the stomatal measurements taken and the lack of extreme variation between stomatal measurements both within the same leaf and the same plant implies that such measurements can be relied upon to be an indication of the ploidy level of the L1 layer of the in vitro plantlets. In addition, many of the plantlets had their stomatal size examined more than once and these stomatal measurements were found to show little or no variation from the original measurements. The only report in the literature, as far as is known, relating to stomatal measurements of Solanum tuberosum is that of Baker (1943), in which stomatal size of tetraploid plants is reported to be 41.44 ± 0.2954μ and that of octoploid plants to be 64.72 ± 0.2557μ. Such figures suggest that stomatal size is not directly proportional to the number of chromosomes, which is also implied by the results obtained through the analysis of both the in vitro and in vivo plants in this study.

Potato, like other plants, has the origin of its adventitious root primordia in regions of the pericycle (Ernst et al., 1968) and therefore a count of the chromosomes in the root-tip should provide sound evidence concerning the ploidy level of the L3 layer. Examination of the chromosome complement of the root-tip cells of the 255 plantlets in vitro revealed the L3 layer of 92% of the plantlets to be tetraploid; of the remainder, 3% of the plantlets appeared to be composed of a diploid L3 layer whereas 5% of the plantlets revealed the presence of aneuploidy through chromosome analysis. Of the 12 plants in which aneuploidy was detected, eight had regenerated from mixed callus systems. As previously stated, chromosomal and stomatal analysis revealed six plantlets to apparently be composed of a diploid L1 layer and a tetraploid L3 layer. Chromosomal analysis of the root-tip cells of these six plantlets once established in vivo confirmed the tetraploid nature of the L3 layer.

All six of the selected regenerants originated from two thin cell layer heterografts made up of donor diploid tissue (S. sparsipilum) grafted onto recipient tetraploid tissue (S. tuberosum ssp. tuberosum cv. Pentland Ivory). Sections 8.5.1 and 8.5.2 describe the external characteristics of the six plantlets under investigation, both in vitro and in vivo. As can be seen from Tables 8.3 and 8.4, there were differences existing between these plantlets and control plants (shoot-tip derived stock material). Dealing first with the in vitro analysis and looking at leaf shape and internode length, the six plantlets resemble S. tuberosum ssp. tuberosum cv. Pentland Ivory more than S. sparsipilum. However, with respect to leaf colour and hair density of the leaves, the six plantlets bear similarity to S. sparsipilum.

It could be argued that any morphological differences exhibited by the six potential chimeras might be the result of the cultural procedure which led to the formation of these plantlets; for example, the tissues within these plantlets were subjected to varying concentrations of different growth regulators over a period of time. This possibility was considered and consequently comparisons were made between the six potential chimeras and plantlets which were derived from autografts of cv. Pentland Ivory and S. sparsipilum. As Table 8.3 shows the autograft-derived plantlets resembled the appropriate controls in leaf shape, leaf colour, hair density and internode length which implied that the differences observed in the heterograft-derived plantlets were not the result of cultural treatment.

Observations were made relating to the type and density of the epidermal hairs, however, it was later decided that these observations were rather subjective and therefore could not be used to assist in the identification of the L1 layer of a plantlet. Leaf shape has been used in work with chimeras as a characteristic that can be used to aid identification of the ploidy level of certain layers. According to Howard (1978), only the epidermis of leaflets traces back to L1 at the growing point, and therefore it would seem that L1 can have very little effect on leaf and leaflet shape. Howard (1978) further reports that most of the tissues of a leaf trace back to L2, but some cells in the central area trace back to L3. However, he does say that it does not necessarily follow that the constitution of L3 has a big effect on leaf and leaflet shape. As the leaves from the six plantlets resembled the simple leaf form of cv. Pentland Ivory rather than the imparipinnate compound leaf form

of S. sparsipilum, it does suggest that the ploidy level of the L2 layer of the six plantlets could be tetraploid. Leaf shape and colour have been used by other workers as corroboratory information as to the ploidy level of the L2 and/or L3 layers (Clayberg, 1974; Sree Ramulu et al., 1976). Nevertheless, it must be remembered that too much reliance on such information can be misleading (Sections 1.2.2 and 8.1). As Table 8.3 shows plantlets numbers two and six were seen to have curled and twisted margins. Neilson-Jones (1969) commenting on the work of Jørgensen et al., (1927) in obtaining chimeras of Solanaceae through grafting in vivo, pointed out that in some of the combinations achieved, the leaves were buckled and malformed. He further added that such a characteristic was most evident when the combination was comprised of plants whose leaves were quite different in shape, such as a small simple leafed plant in combination with a large compound leafed plant. It is likely too that such structural abnormalities could occur as a result of the larger tetraploid cells being contained within an epidermis composed of the smaller diploid cells. As to the other characteristics noted in Table 8.3, although little weight can be attached to them, it is interesting to note that the surface features of the six plantlets are such as would be expected if they possessed a diploid L1 layer.

Table 8.4 shows the morphological characteristics which were examined, once the plants had been established in vivo. Although cv. Pentland Ivory (control) at this stage of development had assumed the imparipinnate compound leaf shape of S. sparsipilum (control) the difference in size and form of the apex was

sufficient to distinguish the two species, and as can be seen from Table 8.4, the leaf shape of the six plantlets still resembled cv. Pentland Ivory (control) more than S. sparsipilum (control). Plant number two was still seen to be showing irregularities around the margin of the leaf; these can be seen quite clearly in Plate 8.6D. Two other morphological characteristics noted in Table 8.4 are the occurrence of tubers in three of the five plantlets (number six died 20 days after transfer to soil), and the slower growth as can be seen by the measurements of height of the leafy shoots. With reference to the latter, Tilney-Bassett (1986) reports that observations of growth retardation and stunting does occur when cytochimeras are induced through the use of colchicine. Although this is partly the result of the toxicity of the colchicine and consequent death of some cells, regular development of plant tissue is likely to be disturbed through the attempted balanced growth of cells of different ploidy. Baker (1943) induced polyploid periclinal chimeras of S. tuberosum cv. Triumph using colchicine, and commented on the vegetative characteristics of these chimeras. The growth of plants of the 8n - 4n type was reduced compared to the untreated plants of the 4n - 4n type but the 4n - 8n plants showed the least development of all parts of the plant. Similarly, Jørgensen et al., (1927) produced a range of Solanum chimeras through grafting in vivo and found that some of the resulting chimeras were characterized by extremely slow growth, at least in the early stages of development.

Analysing potato tubers, cytochimeras, graft chimeras and variegated leaf chimeras has made it evident that potato shoots have a three-layered growing point. However, as discussed in Section 1.2,

it is unclear as to how independent an existence each of these layers has; the main argument being the degree of independence that exists between L2 and L3. In some varieties these two layers remain distinct, however in others, L2 contributes both L2 and L3. Other observations suggest that occasional tangential divisions of cells in L2 may contribute cells to the L1 or L3 layers (Howard, 1971, 1972). Thus it would appear that no one situation is true of all species, and that considerable differences may exist according to the species and cultivar investigated. If one accepts that L3 is dependent on L2, then the chromosome analysis previously discussed, as well as providing evidence concerning the L3 layer also indicates the ploidy level of the L2 layer. However, as there is evidence that the extent of independence between the two layers may vary between different species and cultivars, further analysis supplying additional evidence as to the ploidy level of L2 would be desirable. The original intention in this section of the investigation was to allow the plants to develop to flowering stage and to determine pollen grain size which is supposed to reflect the ploidy level of the L2 layer (Tilney-Bassett, 1986). In addition, the possibility of using the eye-excision method (Asseyeva, 1927) to confirm the constitution of the L3 layer was considered, especially when tuber formation was evident with some of the plants. Unfortunately all the potential chimeral plants died in vivo. As death occurred shortly after greenfly infestation and consequent spraying with an insecticide, actual cause of death was not clear. However, this meant that analysis of the plants could not be completed and thus the true nature of the chimeras remained speculative.

CHAPTER NINE

FINAL DISCUSSION

The aim of this study was to develop an *in vitro* system from which periclinal chimeras of certain potato species could be regenerated. It could be argued that potatoes are suitable candidates for a study of chimeras. Many varieties exist as chimeras, and the majority of these occur where L1 is different from L2 and L3. One well-known commercial variety with such a chimeral structure is cv. Desiree, other examples include cvs. Red King Edward, Golden Wonder, Russet Burbank, to name but a few.(Tilney-Bassett, 1986). Thus the potato plant is amenable to periclinal chimera formation. It is surprising therefore that the outcome of this study was that a total of 2% of the plants regenerated were identified as chimeras on the basis of the analysis performed.

Several explanations exist for the low percentage of chimeras recovered from potential chimeral systems and these have been discussed in Section 1.2.1. The main argument appears to centre around the origin of adventitious shoots, that is, whether or not they are derived from one cell or several cells. No histological evidence has been obtained in this study to favour either argument , however, the existence of even such a small number of chimeras would appear to imply that adventitious shoots can originate from more than one cell. Nevertheless, the production of so few chimeras tends to suggest that, *in vitro*, adventitious shoots are not initially formed from a very large number of cells or that cellular competition (diplontic selection) in newly formed chimeral meristems eliminated one genotype.

If one pursues the argument that adventitious shoots are not derived from a single cell then the next question to arise is one of the cellular origin of adventitious shoots, that is, which layers are involved in adventitious shoot formation. Sree Ramulu et al., (1976) regenerated plants from stem internodal sections of periclinal chimeras of Lycopersicon peruvianum and found that the regenerants originated from the inner histogenic layers L2 and/or L3. Dulieu (1967A; 1967B), in analysing regenerants from periclinal chimeras for chlorophyll deficiency in Nicotiana tabacum found their origins to vary, that is, some were derived from L1 or L2, with a few from L3. Thus it would seem that the cell layers involved in adventitious shoot formation vary with the species, with the individual explant and also within the individual explant. Histological analysis of stem internodal sections (Section 7.4) and thin cell layers (Section 7.6) infers that adventitious shoot formation from these tissues involves cells from L1 and L2 and/or L3. If the number of cell layers taking part in adventitious shoot formation is variable, this implies the importance of factors such as cell communication and cell competence, which would tend to limit the number of potential chimeras which any cell system can regenerate. It could be argued that further evidence for the formation of adventitious shoots from more than one cell and more than one layer is provided by the putative chimeras which regenerated from the thin cell layer graft system in this study. As the shoots regenerating from a system involving diploid donor tissue grafted onto tetraploid recipient tissue were composed of a diploid L1 layer and a tetraploid L3 layer, the implication is that cells responsible for the formation of these adventitious shoot primordia were derived

from at least two layers.

A further factor contributing to the low number of chimeras resulting from this study is likely to be the differences in the regeneration of the two species (S. tuberosum ssp tuberosum cv. Pentland Ivory and S. sparsipilum) used in the thin cell layer heterograft construction. Regeneration studies (Section 7) showed that several differences exist between these two species with respect to shoot regeneration. It is likely that the major factor limiting co-ordinated shoot regeneration from the grafted system was the differing location of shoot regeneration depending on which species the tissue was isolated from. As reported in Section 7.3.2 shoot regeneration from tissue derived from S. sparsipilum is confined to the apical end of the stem internodal explant and neither removal of the epidermal layer (thin cell layer) nor removal followed by replacement of the epidermal layer affects this regeneration site. However, with S. tuberosum ssp tuberosum cv. Pentland Ivory shoot regeneration occurred at both ends of the stem internodal explant though was twice as prolific at the basal end (77%) compared to the apical end (30%). Removal of the epidermal layer or removal followed by replacement of the epidermal layer affected shoot regeneration at both ends of the explant, with the apical end being more affected than the basal end. This distinctive difference in the site of regeneration would hinder the co-ordination likely to be necessary for the formation of an adventitious shoot composed of diploid and tetraploid tissue.

Other regeneration differences noted between the two

species are concerned more with regeneration potential. Culture of stem internodal explants isolated from both species on medium containing zeatin riboside (4ZR) gave optimum shoot regeneration, however those explants isolated from the tetraploid species were less reticent to display their caulogenic potential than those of diploid origin (Section 7.2). Further differences in regeneration potential were observed when epidermal layers (thin cell layers) were excised from stem internodal sections and subjected to culture; such explants isolated from diploid species displayed greater shoot regeneration ability than similar tissues isolated from tetraploid tissue. This differing ability, depending on which part of the stem internode is subjected to culture possibly explains why shoot regeneration occurred when the thin cell layer graft was composed of a responsive diploid thin cell layer grafted onto a responsive tetraploid core. The low percentage of chimeras obtained in this study is not unusual. Carlson et al., (1975) and Marcotrigiano et al., (1984) investigated Nicotiana tabacum in combination with the amphiploid hybrid of N. glauca x N. langsdorfii. Both workers regenerated chimeral shoots from mixed callus systems and achieved success rates of 0.4% and 0.3% respectively. Although it is of an obvious advantage to maximise regeneration from a potential chimeral system, it must be remembered that the construction of a single, chimeral shoot could be sufficient if the species in question is assexually propagated.

As a result of this study six plants were recognised in the in vitro stage as potential chimeras. The evidence in support of this came from stomatal length measurements (providing information

relating to the L1 layer) and chromosome counts of root-tip cells (providing information relating to the L3 layer)(Section 8.2 and 8.3). Of these six plants five were successfully grown on and maintained for approximately 12 weeks in an in vivo environment. Stomatal length and chromosome count analysis confirmed the results obtained in the in vitro stage (Sections 8.6.1 and 8.6.2). Morphological examination appeared to further corroborate these results at both an in vitro level (Section 8.5.1) and an in vivo level (Section 8.5.2). The extent to which this latter evidence can be seen as conclusive has been discussed previously (Section 8.7). From the evidence outlined above it would seem that the five plants reaching the in vivo stage were composed of cells of differing ploidy, in such a way as to infer they were periclinal chimeras.

It could be argued that the results obtained in this study could also point to the plants being mericlinal or sectorial chimeras. If the plants in question were mericlinal or sectorial chimeras, the area of tissue accommodated within the sector or the mericlinal margin would have to be extensive. The basis for this argument lies in the spiral phyllotaxis exhibited by the potato plant and the selection of two leaves (second and third leaves from the apex) for stomatal analysis. In addition, sectorial and mericlinal chimeras tend to be more transient structures whereas periclinal chimeras lend themselves to stability and permanence. The relative stability and permanence of the chimeral state of the plants in this study was confirmed by the constancy of results obtained from the in vitro plantlets and the in vivo plants. The five potential chimeras were characterised by slow growth compared to the growth exhibited by

the control plants (Table 8.4, Section 8.5.2). This growth retardation has been observed by several workers (Jørgensen et al., 1927; Baker, 1943; Tilney-Bassett, 1986) and therefore it could be that this is further evidence that the plants in question were of chimeral composition.

These chimeras which regenerated from the thin cell layer heterografts could have resulted from experimental treatment or from chromosomal alterations. As discussed in Section 1.5, regenerating plants adventitiously within a tissue culture system can present problems where chromosomal stability is concerned. Compared with mutations occurring in vivo, cells growing in vitro show comparably higher rates of nuclear abnormalities, especially changes in chromosome number (D'Amato, 1978). Although chimeras can come about through loss or gain of individual chromosomes or chromosome fragments owing to misdivision, they do not usually develop into stable chimeras (Tilney-Bassett, 1986). More commonly, cytochimeras have simple multiples of the normal chromosome complement in the changed layers. As the techniques utilised in this investigation necessitated the use of several growth regulators over a long period of time, the possibility cannot be ruled out that such conditions influenced the normal course of mitoses, thus creating cytochimeras. Horak (1972) argues that the spontaneous occurrence of chromosome chimeras arising from differing tissue culture systems is rare; in his work with kale he identified two of the 17 regenerants as sectorial chimeras. Sacristan et al., (1969) found four mixochimeral plants out of 142 regenerants derived from the Tc line of tumour tissues. Considering the low number of chimeras which are induced as a

result of experimental treatment (for example, Marcotrigiano et al., (1984) acknowledged four chimeras from 1321 regenerants, and in this study only six of the 255 plantlets examined were identified as possible chimeras), it would seem that there is little difference between the numbers of chimeras arising as a result of deliberate experimental treatment and the numbers which are formed due to spontaneous occurrence within a tissue culture system. Consequently it might be argued that no conclusive decision can be made as to what instigated chimera formation in this study. On the other hand, it could be claimed that if tissue culture systems were highly supportive of chimera formation then their regeneration within such systems would be a more regular occurrence; it might be that they do occur but are either recognized and not reported, or are not recognized. Several workers have commented on the absence of chimeras in their tissue culture systems for example, Jacobsen (1981) in investigating a tissue culture method aimed at inducing polyploidization in regenerated plants from leaf callus of dihaploid potato, found no trace of chimeras, even though he managed to produce plants of different ploidy levels. What seems likely is that although tissue culture conditions can invoke chromosomal changes leading to systems containing cells of different chromosome complements, the communication and co-ordination necessary for chimeral formation to take place reduces to a large degree the number of chimeras which do result. Ball (1969) puts forward a similar proposal when he poses the question "What are the additional and/or chemical factors necessary to the formation of chimeras beyond the mere adjacency of the cells of two component tissues"?

Some indication of chromosomal change in the chimeral systems investigated in this study was provided by root-tip chromosome counts (Section 8.3); these identified the presence of plants showing aneuploidy in the L3 layer. However the percentage of plants detected which exhibited aneuploidy was low (5%). Of the 12 plants showing aneuploidy in the L3 layer, nine were derived from a mixed callus system. Thus it would seem that of the two chimeral systems investigated the mixed callus system favoured chromosomal change rather than the thin cell layer graft system. Therefore if the regenerants of chimeral composition were the result of spontaneous chromosomal change rather than experimental treatment, one would surely expect them to have been the products of the mixed callus system rather than the thin cell layer graft system. Furthermore, as the thin cell layer graft system would appear to be relatively stable with respect to chromosome number, it is more likely that the chimeral regenerants arising from this system were the result of experimental treatment rather than a spontaneous change in chromosome number. A further consideration is that as only two thin cell layer heterografts gave rise to chimeral regenerants, this suggests again that there are far more exacting requirements for chimeral formation than the presence of cells in a system undergoing chromosomal change.

Evaluation of the two systems as to their potential for producing chimeras points to that of the thin cell layer graft system as being preferable to that of the mixed callus system. However, this system requires greater skill and is more time-consuming than the mixed callus system. Of the two systems, the mixed callus

system is more likely to create problems where genetic stability is concerned, as a result of the extended callus phase, which is a necessary part of the mixed callus procedure. Both systems appear to have problems associated with regeneration, however experiments carried out during this study have identified several factors which contributed to this reticence, for example, the carry-over of growth regulators from the grafting medium. It is therefore likely that regeneration levels in the future could be improved. Having said this, it must be remembered (as previously mentioned) that as potato is asexually propagated, the construction of a single, stable chimeral plant could be sufficient.

This study has shown that chimera formation is possible between two potato species. With the original aim of the study in mind, it would be interesting to attempt to construct a thin cell layer heterograft between a high-yielding potato variety, and the potato species, Solanum berthaultii, reputed to be resistant to a wide range of pests as a result of the multitude of glandular trichomes within its epidermis. The chimera originating from such a combination would then need full evaluation as to its pest resistance properties.

APPENDIX I

MEDIA AND REAGENTS

I.I. BASAL CULTURE MEDIUM (SECTION 2.2)

CONSTITUENT	mgl^{-1}	mM
NH_4NO_3	1650	48.5
KNO_3	1900	18.8
$CaCl_2.2H_2O$	440	3.0
$MgSO_4.7H_2O$	370	1.5
KH_2PO_4	170	1.25
Na_2EDTA	37.3	0.2
$FeSO_4.7H_2O$	27.8	0.1
H_3BO_3	6.2	0.1
$MnSO_4.4H_2O$	22.3	0.1
$ZnSO_4.6H_2O$	8.6	0.03
KI	0.83	5×10^{-3}
$Na_2MoO_4.2H_2O$	0.25	1×10^{-3}
$CuSO_4.5H_2O$	0.025	0.1×10^{-3}
$CoSO_4.6H_2O$	0.025	0.1×10^{-3}
Glycine	2.0	0.03
Nicotinic Acid	0.5	4×10^{-3}
Pyridoxine HCl	0.5	2.4×10^{-3}
Thiamine HCl	0.1	0.3×10^{-3}
Myo-inositol	100	0.55
Agar	$8gl^{-1}$	
pH	5.7	
Reference	Murashige & Skoog, 1962	

I.2. CALLUS INDUCTION AND MAINTENANCE MEDIUM - ST. (SECTION 4.3)

CONSTITUENT	mgl^{-1}	mM
KNO_3	1900	18.8
$CaCl_2.2H_2O$	440	3.0
$MgSO_4.7H_2O$	370	1.5
KH_2PO_4.	170	1.25
Na_2EDTA	37.3	0.2
$FeSO_4.7H_2O$	27.8	0.1
H_3BO_3	6.2	0.1
$MnCl_2.4H_2O$	19.8	0.1
$ZnSO_4.7H_2O$	9.2	3×10^{-2}
KI	0.83	5×10^{-3}
$Na_2MoO_4.2H_2O$	0.25	1×10^{-3}
$CuSO_4.5H_2O$	0.025	0.1×10^{-3}
$CoSO_4.7H_2O$	0.030	0.025×10^{-3}
Myo-inositol	100	0.55
Thiamine-HCl	0.5	1.5×10^{-3}
Glycine	2.0	0.03
Nicotinic Acid	5.0	4×10^{-2}
Pyridoxine-HCl	0.5	2.4×10^{-3}
Folic acid	0.5	1.1×10^{-3}
Biotin	0.05	2×10^{-4}
Casein hydrolysate	1000	-
Adenine sulphate	40	-
1-Napthaleneacetic acid	0.05	2.7×10^{-4}
6-Benzylaminopurine	0.5	2.2×10^{-3}

I.2. (CONTINUED). CALLUS INDUCTION AND MAINTENANCE MEDIUM - ST.

(SECTION 4.3)

CONSTITUENT	mgl^{-1}	mM
D-Mannitol	$54.66gl^{-1}$	0.3M
Sucrose	$5gl^{-1}$	15mM
2-N-Morpholino-ethane sulphonic acid		5.0
Agar	$8gl^{-1}$	
pH	5.8	
Reference	Shepard & Totten, 1977	

I.3. CALLUS REGENERATION MEDIA - D1 and D2 (SECTION 7.13)

CONSTITUENT	MEDIUM D1		MEDIUM D2	
	Mgl^{-1}	mM	Mgl^{-1}	mM
NH_4Cl	107	2.0	267.5	5.0
KNO_3	1900	18.8	1900	18.8
$CaCl_2.2H_2O$	440	3.0	440	3.0
$MgSO_4.7H_2O$	370	1.5	370	1.5
KH_2PO_4	170	1.25	170	1.25
Na_2EDTA	18.5	4.9×10^{-2}	18.5	4.9×10^{-2}
$FeSO_4.7H_2O$	13.9	4.9×10^{-2}	13.9	4.9×10^{-2}
H_3BO_3	3.1	5.0×10^{-2}	3.1	5.0×10^{-2}
$MnCl_2.4H_2O$	9.9	5.0×10^{-2}	9.9	5.0×10^{-2}
$ZnSO_4.7H_2O$	4.6	1.6×10^{-2}	4.6	1.6×10^{-2}
KI	0.42	2.5×10^{-3}	0.42	2.5×10^{-3}
$Na_2MoO_4.2H_2O$	0.13	5.4×10^{-4}	0.13	5.4×10^{-4}
$CuSO_4.5H_2O$	0.013	5.2×10^{-5}	0.013	5.2×10^{-5}
$CoSO_4.7H_2O$	0.015	5.3×10^{-5}	0.015	5.3×10^{-5}
Myo-inositol	100	0.55	100	0.55
Thiamine-HCl	0.5	1.5×10^{-3}	0.5	1.5×10^{-3}
Glycine	2.0	0.03	2.0	0.03
Nicotinic acid	5.0	4×10^{-2}	5.0	4×10^{-2}
Pyridoxine-HCl	0.5	2.4×10^{-3}	0.5	2.4×10^{-3}
Folic acid	0.5	1.1×10^{-3}	0.5	1.1×10^{-3}
Biotin	0.05	2×10^{-4}	0.05	2×10^{-4}
Casein hydrolysate	100	-	100	-
Adenine sulphate	40	-	80	-
1-Napthaleneacetic acid	0.1	5.4×10^{-4}	-	5.4×10^{-4}
Indole-3-acetic acid	-	-	0.1	5.7×10^{-4}
6-Benzylaminopurine	0.5	2.22×10^{-3}	-	-
Zeatin	-	-	1.0	4.6×10^{-3}

1.3. (CONTINUED). CALLUS REGENERATION MEDIA - D1 AND D2 (SECTION 7.13)

CONSTITUENT	MEDIUM D1		MEDIUM D2	
	Mgl^{-1}	mM	Mgl^{-1}	mM
D-Mannitol	$54.66gl^{-1}$	0.3M	$36.44gl^{-1}$	0.2M
Sucrose	$0.25gl^{-1}$	7.3×10^{-4}	$2.5gl^{-1}$	7.3×10^{-3}
2-N-Morpholino-ethane sulphonic acid		5.0		5.0
Agar	$8gl^{-1}$		$8gl^{-1}$	
pH	5.6		5.6	
Reference:	Shepard (1980) but with modifications recommended by Nelson et al., (1983)			

1.4. CALLUS REGENERATION MEDIUM - SCH (SECTION 7.13)

CONSTITUENT	Mgl^{-1}	mM
NH_4NO_3	1650	48.5
KNO_3	1900	18.8
$CaCl_2.2H_2O$	440	3.0
$MgSO_4.7H_2O$	370	1.5
KH_2PO_4	170	1.25
Na_2EDTA	37.3	0.2
$FeSO_4.7H_2O$	27.8	0.1
H_3BO_3	6.2	0.1
$MnSO_4 4H_2O$	22.3	0.1
$ZnSO_4.6H_2O$	8.6	0.03
KI	0.83	5×10^{-3}
$Na_2MoO_4.2H_2O$	0.25	1×10^{-3}
$CuSO_4.5H_2O$	0.025	0.1×10^{-3}
$CoSO_4.6H_2O$	0.025	0.1×10^{-3}
Myo-inositol	100	0.55
Glycine	2.0	0.03
Nicotinic acid	0.5	4×10^{-3}
Pyridoxine-HCl	0.5	2.4×10^{-3}
Thiamine-HCl	0.1	0.3×10^{-3}
Casein hydrolysate	1000	-
Adenine sulphate	40	-
Indole-3-acetic acid	0.1	5.7×10^{-4}
Zeatin	2.0	9.1×10^{-3}
Gibberellic acid	0.1	2.9×10^{-4}
D-Mannitol	$36.44 gl^{-1}$	0.2
Sucrose	$5 gl^{-1}$	15mM
pH	5.8	
Reference	Schumann et al., 1980.	

Appendix I.4

Staining plant tissues by Feulgen reaction

Schiff's reagent

Basic fuchsin	0.7g
Sodium metabisulphite	3.8g
0.15N Hydrochloric acid	$200cm^3$
Activated charcoal	1.0g
pH	2.2

Fuchsin and sodium metabisulphite were added to $200cm^3$ of hydrochloric acid (0.15N) and this solution was stirred for two hours at 20^oC. Activated charcoal was used to decolourise the solution, which was then filtered and the volume made up to $200cm^3$ with distilled water.

Reference: Feulgen et al., 1924

The above reagent was used in Section 8.2 to determine the chromosome number of root-tips of plantlets cultured in vitro. The same reagent was also used in Section 5.4 to ascertain the nuclear DNA content of callus cells.

APPENDIX II

DATA

Appendix II.I

A. The growth of callus derived from stem tissue of *S. sparsipilum* when cultured in isolation or in combination with *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory on a medium supplemented with 2,4-D ($2.0 mgl^{-1}$)(Table 5.3, Section 5.3)

CALLUS SYSTEM	FRESH WEIGHT INCREASE (G) AFTER ONE MONTH'S CULTURE
In isolation (single)	0.37, 0.61, 0.209, 0.622, 0.558, 0.451, 0.453, 0.567, 0.664, 0.619, 0.221, 0.541, 0.369, 0.402, 0.516, 0.609.
In combination (mixed)	0.457, 0.427, 0.464, 0.574, 0.491, 0.423, 0.489, 0.630, 0.509, 0.640, 0.362, 0.656, 0.422, 0.381, 0.336, 0.332, 0.283, 0.285
	FRESH WEIGHT INCREASE (G) AFTER TWO MONTH'S CULTURE
In isolation (single)	1.081, 0.961, 0.979, 0.948, 0.996, 1.046, 0.977, 1.261, 1.262, 1.571, 1.091, 1.211, 0.956, 1.241, 0.942, 1.055, 1.089, 1.044
In combination (mixed)	0.937, 0.799, 0.945, 0.930, 0.973, 0.987, 1.058, 0.813, 0.809 0.907 0.863, 1.029, 1.058, 0.925, 0.883, 0.947, 0.887, 0.869.

Appendix II.I (continued)

B. The growth of callus derived from stem tissue of S. tuberosum ssp. tuberosum cv. Pentland Ivory when cultured in isolation or in combination with S. sparsipilum on a medium supplemented with 2,4-D ($2.0 mg l^{-1}$)(Table 5.3, Section 5.3)

CALLUS SYSTEM	FRESH WEIGHT INCREASE (G) AFTER ONE MONTH'S CULTURE
In isolation (single)	1.095, 1.080, 1.099, 1.039, 1.145, 1.017, 1.111, 1.140, 1.187, 1.053, 1.170, 0.996, 1.053, 0.987, 1.041, 0.916, 1.174, 1.132, 1.127, 0.962
In combination (mixed)	0.753, 0.809 0.705, 0.784, 0.738, 0.787, 0.802, 0.756, 0.699, 0.754, 0.749, 0.866, 0.777, 0.710, 0.802, 0.855 0.812 0.882
	FRESH WEIGHT INCREASE (G) AFTER TWO MONTH'S CULTURE
In isolation (single)	1.999, 2.022, 1.967, 1.876, 1.878, 2.201, 2.162, 2.109 2.030, 2.143, 2.009, 2.015, 1.804, 2.091, 1.885, 2.006, 2.096, 1.959, 1.916, 1.892.
In combination (mixed)	1.641, 1.458, 1.267, 1.201, 1.525, 1.243, 1.647, 1.326, 1.638, 1.486, 1.523, 1.355, 1.313, 1.341, 1.255, 1.370.

Appendix II.2.

Nuclear DNA absorption values obtained from callus induced from stem tissue of S. sparsipilum (see Section 5.4)

A. Callus cultured for three months on basal culture medium supplemented with 3% (w/v) sucrose and 2,4-D ($2.0 mgl^{-1}$)(Section 4.2)

27.7	29.0	27.6	25.1	25.8	24.3	26.6	26.1	22.8	16.1	25.7	20.6
19.3	22.3	20.3	15.5	21.3	11.2	34.1	21.5	20.6	23.7	26.4	18.5
15.7	33.5	19.3	23.0	29.4	19.2	28.4	28.9	36.7	31.0	28.2	22.8
26.8	16.8	33.9	17.4	31.0	20.6	24.6	31.8	14.3	13.5	11.7	24.9
17.0	19.0	19.0	33.6	19.6	29.9	31.1	34.0	14.8	21.3	31.0	26.7
17.6	15.0	18.4	28.2	28.1	19.2	23.5	28.6	25.0	22.0	13.4	26.4
26.5	30.8	20.3	17.9	22.2	25.7	25.0	16.4	12.6	25.3	20.0	15.0
15.7	15.8	13.4	15.8	19.3	21.9	19.4	14.0	22.1	22.2	19.6	14.8
16.2	20.3	21.6	15.2	17.1	19.2	22.9	26.3	17.3	18.1	28.5	16.2
17.5	15.0	25.3	22.5	16.9	26.1	26.4	12.6	20.5	15.7	25.6	21.6

These figures have been plotted in histogram form (see Fig. 5.2A, Section 5.4)

Appendix II.2. (continued)

B. Callus cultured for 13 months on basal culture medium supplemented with 3% sucrose (w/v) and 2,4-D ($2.0 mgl^{-1}$) (see Section 4.2)

21.8	16.4	23.4	21.4	12.5	18.5	23.7	21.5	24.8	14.8	12.7	17.0
12.1	22.7	21.9	26.9	18.3	19.7	17.7	16.8	16.3	11.3	10.9	19.0
26.4	19.6	17.5	33.2	20.1	12.1	12.2	10.3	11.3	10.2	10.7	21.4
10.5	21.1	10.4	17.7	15.7	17.2	18.9	14.7	20.5	12.5	12.5	12.7
10.3	10.4	15.7	18.7	19.0	10.2	10.5	13.0	12.6	13.2	11.4	12.6
13.1	16.9	11.9	20.8	11.2	12.1	22.0	16.6	13.6	11.9	21.9	12.1
13.5	9.5	18.8	18.4	10.0	10.2	24.2	12.6	14.6	32.4	27.3	24.0
31.8	11.4	13.5	9.4	17.4	16.1	9.5	10.8	9.5	15.5	16.7	13.4
9.5	14.9	12.2	11.0	15.7	16.1	12.1	17.4	22.6	22.3	21.6	19.7
16.3	27.2	21.6	12.9	25.3	14.6	15.2	25.2	16.7	21.7	26.3	24.9
27.2	16.9	15.3	26.4	21.9	12.6	22.2	21.6	16.7	29.6	28.2	15.3
17.1	26.7										

These figures have been plotted in histogram form (see Fig. 5.2B, Section 5.4)

Appendix II.2 (continued)

C. Nuclear DNA absorption values obtained from callus induced from stem tissue of S. tuberosum ssp tuberosum cv. Pentland Ivory (see Section 5.4)

Callus cultured for three months on basal culture medium supplemented with 3% sucrose (w/v) and 2,4-D ($2.0 mg l^{-1}$) Section 4.2)

48.9	44.5	45.3	49.3	38.7	82.9	51.2	52.7	42.1	80.1	49.9	57.3
49.2	82.8	50.5	88.3	52.6	46.1	54.2	88.2	55.5	50.4	51.9	50.6
57.4	58.3	45.0	44.9	35.2	53.1	58.1	58.2	56.1	61.2	60.5	59.2
53.6	42.1	40.6	50.0	46.4	50.6	42.1	50.7	53.3	50.0	47.8	42.9
56.7	54.7	65.7	58.4	53.4	50.3	50.1	49.6	46.3	50.0	53.0	47.3
48.6	37.9	37.5	77.7	45.0	47.8	50.2	81.0	52.6	49.8	50.9	55.9
55.7	58.2	52.3	63.6	65.6	53.7	40.9	40.8	39.7	44.5	45.7	40.7
37.4	43.2	39.7	44.4	51.1	77.0	48.3	57.7	45.7	42.9	71.1	42.8
42.7	38.7	41.5	46.2	43.0	38.1	86.2	41.2	46.2	51.3	42.6	41.7
39.8	52.3	56.7	51.4	43.2	51.2	40.5	51.6	49.2	53.2	49.2	50.8
51.8	59.2	45.1	55.4	41.3	43.9	52.6	55.6	50.6	45.4	47.5	47.0

These figures have been plotted in histogram form (see Fig. 5.2C, Section 5.4)

Appendix II.2. (continued)

D. Nuclear DNA absorption values obtained from callus induced from stem tissue of S. tuberosum ssp. tuberosum cv. Pentland Ivory (Section 5.4)

Callus cultured for 13 months on basal culture medium supplemented with 3% sucrose (w/v) and 2,4-D ($2.0 mgl^{-1}$)(Section 4.2)

36.9	42.3	45.6	41.7	51.7	42.6	33.9	72.1	83.7	52.6	67.9	62.8
45.0	49.6	53.2	55.5	61.9	32.6	61.2	55.3	49.8	46.9	42.1	43.4
46.7	46.3	51.2	73.3	64.7	82.9	64.4	35.6	77.1	46.9	53.2	54.5
57.8	31.9	86.2	61.9	46.7	53.9	54.2	40.9	45.7	46.8	49.2	69.3
46.8	49.8	53.7	59.2	57.8	86.3	46.9	45.3	56.7	58.9	59.8	63.6
81.2	52.6	51.9	82.9	77.6	56.7	42.4	49.8	56.2	41.3	59.8	71.6
52.6	59.8	53.6	42.9	41.8	86.2	66.6	43.2	44.4	45.6	49.8	42.3
63.3	42.6	51.3	52.6	55.5	57.8	59.8	56.7	56.6	41.2	49.3	58.7
63.3	41.9	52.6	55.7	89.9	52.7	51.9	42.3	46.7	57.7	41.9	55.6
41.3	54.4	53.6	41.2	44.4	43.6	46.6	49.0	55.6	51.8	42.0	41.0

These figures have been plotted in histogram form (see Fig. 5.2D, Section 5.4)

Appendix II.2. (continued)

E. Nuclear DNA absorption values obtained from a mixed callus system consisting of callus induced from stem tissue of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory (Section 5.4)

Callus cultured for nine months: mixed system (OT) with cv. Pentland Ivory as the uppermost partner (Section 5.2)

20.7	37.4	23.9	20.5	23.8	14.2	45.1	37.7	30.0	24.7	24.5	27.1
29.8	26.3	27.4	26.6	25.5	26.5	26.6	29.1	32.4	30.1	40.1	32.6
26.5	25.6	26.1	24.8	29.2	25.7	35.0	25.6	25.6	24.0	26.4	25.5
24.8	24.6	24.9	75.0	71.8	63.1	58.6	20.5	20.3	18.5	18.1	18.3
14.7	17.1	20.7	41.0	38.0	44.2	70.0	73.4	35.2	30.1	36.1	33.5
30.3	26.4	34.9	45.1	36.6	21.4	29.5	27.5	34.9	51.1	49.2	52.5
48.5	65.6	28.1	31.9	32.2	40.7	51.5	12.4	10.2	12.0	12.5	13.8
19.8	16.3	16.1	65.1	30.2	19.5	15.8	47.5	45.3	30.0	17.9	40.6
11.5	12.2	10.8	19.8	25.1	17.0	25.2	35.2	19.5	20.7	17.6	18.5
33.4	60.8	11.2	84.3	33.8	48.9	16.7	24.0	26.9	31.3	29.8	32.2
26.8	64.6	33.3	36.2	24.2	29.8	26.6	35.1	26.9	32.3	24.4	26.8

These figures have been plotted in histogram form (see Fig, 5.2E, Section 5.4)

Appendix II.2 (continued)

F. Nuclear DNA values obtained from a mixed callus system consisting of callus induced from stem tissue of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory (Section 5.4)

Callus cultured for nine months: mixed system (OT) with cv. Pentland Ivory as the uppermost partner (Section 5.2)

10.3	17.8	20.2	15.9	18.7	17.4	20.3	22.5	13.3	34.2	32.4	18.1
8.8	35.6	36.6	35.2	33.7	66.7	44.9	47.9	30.6	67.4	21.6	23.3
23.1	32.6	35.5	36.5	23.4	31.9	37.3	58.8	29.1	33.4	49.1	35.4
27.8	32.0	27.4	15.5	27.5	33.8	21.8	20.9	19.4	35.7	15.9	33.6
22.0	10.8	26.9	34.8	29.3	42.3	45.4	18.3	32.8	12.6	22.4	13.5
10.1	19.4	29.8	19.6	41.7	64.4	14.5	15.5	23.3	27.8	22.9	31.4
27.5	34.7	30.4	34.1	42.5	25.6	12.2	12.9	30.0	30.9	39.3	14.7
21.1	12.5	12.3	13.1	21.4	24.6	35.6	14.3	25.0	30.7	16.9	34.9
37.1	20.4	22.0	46.3	25.3	11.0	15.3	16.2	15.8	15.9	17.4	14.7
15.7	18.3	15.8	15.8	15.6	16.6	17.3	16.4	15.3	9.5	24.0	42.8
20.4	14.6	16.5	19.5	30.5	13.9	9.9	20.2	10.2	10.2	23.5	22.6

These figures have been plotted in histogram form (see Fig. 5.2F, Section 5.4)

Appendix II.2 (continued)

G. Nuclear DNA values obtained from a mixed callus system consisting of callus induced from stem tissue of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory (Section 5.4) cv. Pentland Ivory and S. sparsipilum co-cultured (MC) for nine months (Section 5.2)

26.4	28.3	57.3	46.5	51.7	60.4	59.4	70.9	40.0	49.7	25.3	39.0
35.9	70.4	88.8	76.3	58.3	40.3	32.9	25.4	25.8	30.9	43.7	41.4
38.4	46.0	46.9	24.1	21.4	55.0	29.2	25.8	37.3	50.1	43.4	28.0
39.5	45.2	48.0	24.4	20.4	24.2	24.4	29.0	40.9	40.5	24.7	35.1
62.0	61.7	71.3	58.7	58.2	38.1	31.3	16.7	29.5	33.6	54.8	46.6
47.5	62.8	33.3	28.0	32.8	56.0	29.1	34.2	33.8	46.8	27.2	29.0
30.7	33.6	37.5	24.8	27.6	58.3	62.8	36.7	20.7	35.9	34.0	31.4
31.2	24.7	46.4	37.1	41.6	23.7	53.3	51.1	79.2	88.2	62.9	54.3
52.7	51.0	83.4	84.5	34.1	22.3	32.5	29.3	57.8	83.8	77.7	50.0
46.5	82.0	40.5	23.3	22.6	25.3	34.4	54.1	29.9	39.9	35.7	30.4
48.1	15.3	42.2	50.2	49.4	59.4	33.6	25.0	26.2	27.6	23.2	43.5
23.5	22.0	19.3	46.5	52.4	38.2	41.9	35.2	20.9	16.3	26.8	33.4

These figures have been plotted in histogram from (see Fig. 5.2G, Section 5.4)

Appendix II.2 (continued)

H. Nuclear DNA values obtained from a mixed callus system consisting of callus induced from stem tissue of *S. sparsipilum* and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory (Section 5.4)

A sample of callus as indicated by point I (Fig. 5.I., Section 5.4) from mixed callus of cv. Pentland Ivory and *S. sparsipilum* co-cultured (SS) for four months (Section 5.2)

49.5	45.4	59.0	21.2	78.7	29.0	25.7	38.8	38.1	47.3	53.1	51.1
88.4	46.9	53.3	80.4	77.3	76.1	28.4	68.9	64.0	48.2	45.2	62.1
38.2	36.4	42.9	41.3	89.7	65.2	84.7	86.8	49.1	70.3	68.6	68.4
84.2	87.2	66.5	54.7	59.4	73.8	45.1	88.4	63.0	72.5	77.1	78.4
73.8	75.6	75.9	78.4	73.8	75.6	75.9	88.3	70.3	38.4	65.6	56.0
36.4	57.5	26.5	80.7	60.6	80.8	78.5	68.7	81.3	79.9	82.3	61.0
55.8	59.9	59.8	69.0	67.7	62.5	23.0	46.1	36.3	30.7	36.8	27.8
42.5	73.7	55.5	40.1	86.8	83.6	56.3	71.0	43.9	77.8	89.0	85.0
77.6	53.9	77.2	35.3	67.0	59.3	70.4	49.6	48.4	27.1	32.7	19.0
77.1	20.7	44.9	45.3	30.8	34.5	32.7	30.6	22.1	44.9	31.2	23.9
25.2	24.8	76.3	18.3	65.2	86.4	77.6	69.8	70.4	76.3	49.3	44.2

These figures have been plotted in histogram form (see Fig. 5.2H, Section 5.4)

Appendix II.2. (continued)

I. Nuclear DNA values obtained from a mixed callus system consisting of callus induced from stem tissue of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory (Section 5.4)

A sample of callus as indicated by point 2 (Fig. 5.I. Section 5.4) from mixed callus of cv. Pentland Ivory and S. sparsipilum co-cultured (SS) for four months (Section 5.2)

69.2	74.3	61.9	35.4	82.5	80.2	82.5	49.4	48.3	47.9	89.5	75.2
66.5	42.3	84.5	39.6	70.8	65.0	76.6	70.6	66.6	40.7	61.9	74.4
70.4	81.3	72.5	72.6	60.7	61.3	37.8	28.9	25.6	24.7	28.2	52.8
68.9	78.3	38.8	56.2	60.1	72.7	48.2	78.4	43.5	80.9	65.1	61.6
84.9	52.8	67.9	71.0	75.2	41.9	74.5	61.9	67.7	72.4	61.9	70.9
86.6	45.7	78.6	71.6	35.6	83.9	88.7	89.2	85.1	82.3	83.1	74.3
50.7	48.1	54.4	80.7	82.2	87.8	74.3	68.3	76.9	76.1	86.9	69.5
79.5	58.0	53.6	52.5	60.9	49.9	51.0	60.0	51.3	85.4	57.6	51.9
49.5	55.6	52.7	50.6	74.7	57.7	46.0	44.3	29.6	26.0	87.5	52.0
50.3	45.6	42.8	49.0	32.3	44.7	51.3	27.0	36.7	33.2	36.9	81.6
42.5	61.6	70.3	72.7	66.3	80.2	81.1	71.8	43.3	58.9	52.8	49.8

These figures have been plotted in histogram form (see Fig. 5.2I, Section 5.4)

Appendix II.2 (continued)

J. Nuclear DNA values obtained from a mixed callus system consisting of callus induced from stem tissue of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory (Section 5.4)

A sample of callus as indicated by point 3 (Fig. 5.I, Section 5.4) from mixed callus of cv. Pentland Ivory and S. sparsipilum co-cultured (SS) for four months (Section 5.2)

77.3	42.7	39.8	46.2	63.6	36.5	52.0	54.2	72.3	57.3	67.2	32.5
33.1	38.0	41.8	41.2	70.3	38.2	31.5	36.7	59.8	55.8	68.4	
44.5	47.4	34.6	46.8	47.6	33.6	51.7	48.5	41.2	72.7	46.9	48.9
45.3	73.1	74.3	69.0	66.0	82.4	85.1	68.0	61.3	79.8	71.6	38.2
35.1	50.1	35.1	42.0	59.8	37.6	36.2	29.6	46.4	47.1	29.1	47.6
43.9	39.6	42.2	59.2	77.0	57.9	45.7	80.4	64.7	21.3	31.1	31.5
28.6	27.7	30.3	32.5	27.7	20.3	29.4	30.5	33.2	32.6	24.0	29.4
37.7	47.4	37.2	35.2	50.1	56.2	53.0	45.8	56.2	53.9	27.4	25.5
38.6	50.8	24.3	37.7	38.0	54.9	49.6	33.7	35.3	39.2	35.4	45.8
71.7	31.9	55.9	34.8	33.3	45.0	31.2	32.5	29.8	37.1	47.0	69.5
38.7	35.5	69.2	70.0	73.8	74.2	42.5	42.5	37.2	47.3	31.9	39.5

These figures have been plotted in histogram form (see Fig. 5.2J, Section 5.4)

Appendix II.3. Morphogenetic response of thin cell layer heterografts after culture on regeneration media

(Fig. 7.1, Section 7.7)

TYPE OF HETERO-GRAFT	TWO-STAGE PROCEDURE E31/E41			TWO-STAGE PROCEDURE E32/E42			REGENERATION MEDIUM 4ZR		
	% EXPLANTS WITH CALLUS	% EXPLANTS WITH SHOOTS	% EXPLANTS WITH ROOTS	% EXPLANTS WITH CALLUS	% EXPLANTS WITH SHOOTS	% EXPLANTS WITH ROOTS	% EXPLANTS WITH CALLUS	% EXPLANTS WITH SHOOTS	% EXPLANTS WITH ROOTS
Donor tissue grafted onto tetraploid tissue (2n→4n)	91	-	20	93	4	22	100	24	31
Tetraploid tissue grafted onto donor tissue (4n→2n)	100	-	19	97	-	44	100	9	76

Media: MS + 2% sucrose (w/v) + various combinations of different growth regulators

Temperature: 22 ± 1°C

Light conditions: 16h daylength; 85$\mu Mm^{-2}S^{-1}$PAR

Period of culture: 6 months

Number of replicates per treatment: E31/E41 - 77; E32/E42 - 77; 4ZR - 79

Appendix II.4.

A. Stomatal guard cell length measurements obtained from the leaves of plantlets regenerated from heterografts (HG) and mixed callus systems consisting of tissue derived from *S. sparsipilum* and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory (Section 8.2)

ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)	ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)
HG 4n→2n	28.0	HG 2n→4n	33.62
"	27.62	"	34.50
"	28.37	"	39.62
"	29.37	HG 4n→2n	35.62
"	30.87	"	48.62
"	27.25	"	35.75
"	28.37	"	34.12
"	28.00	"	36.69
HG 2n→4n	44.62	"	34.87
"	31.75	HG 2n→4n	31.0
"	39.62	"	33.33
"	47.67	"	34.50
"	35.62	"	31.37
"	37.50	"	32.87
"	38.37	"	34.37
"	34.87	"	30.89
"	41.87	"	32.25
"	33.62	"	32.62
"	38.12	"	34.75
"	40.62	"	31.50
"	38.12	"	33.37
"	34.50	"	34.50
"	35.37	"	41.37
"	35.37	"	41.87
"	34.62	"	32.00
"	42.50	"	35.36
"	36.87	"	33.67
"	33.00	"	34.17

Appendix II.4. (continued)

	ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)	ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)
	HG 2n→4n	31.50	HG 2n→4n	34.00
	"	33.87	"	33.83
	"	33.75	"	36.44
	"	34.62	"	33.86
	"	35.86	"	32.92
*	"	33.71	"	43.00
	"	32.18	"	37.83
	"	37.33	"	32.33
	"	32.67	"	32.17
	HG 4n→2n	44.50	"	31.83
	"	40.67	"	33.67
	"	37.50	"	33.17
	"	40.00	"	33.67
	"	39.00	"	31.67
	"	28.26	"	43.00
	"	33.83	"	36.83
	HG 2n→4n	39.00	"	38.33
	"	37.83	"	41.50
*	"	37.67	"	33.67
	"	31.33	"	32.67
	"	33.00	"	35.33
	"	32.23	"	33.83
	"	40.50	"	34.83
*	"	37.83	"	35.83
	"	32.83	"	44.33
	"	38.50	"	37.43
	"	36.33	"	41.71
	"	34.66	* "	42.86
	"	34.17	"	37.00
	"	34.33	"	37.00
	"	32.83	"	38.14
	"	32.93	"	41.28

Appendix II.4 (continued)

ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)	ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)
HG 2n→4n	35.67	HG 2n→4n	40.43
"	39.62	"	41.14
"	39.75	"	42.71
"	36.50	"	38.28
"	34.12	"	37.26
"	32.37	"	41.57
"	28.67	"	40.14
"	27.50	"	33.57
"	37.06	"	37.57
"	32.00	"	34.00
"	37.00	"	37.43
"	42.00	"	31.43
"	35.00	"	43.62
"	35.12	"	44.50
"	35.06	"	32.00
"	40.02	"	40.00
"	39.12	"	40.62
"	42.85	"	46.25
"	42.14	"	41.12
"	34.28	"	44.37
"	34.14	"	35.12
"	35.71	"	36.12
"	42.62	*Mixed callus	45.24
"	44.12	"	44.09
"	31.33	"	42.16
"	35.87	"	43.33
"	39.37	"	40.12
"	33.87	"	41.24
"	34.00	* "	40.26
"	35.17	"	42.25
"	37.00	"	42.16
"	33.37	"	40.19
"	42.00	"	42.25

Appendix II.4. (continued)

ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)	ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)
HG 2n→4n	44.25	Mixed callus	44.86
"	42.87	"	41.71
"	42.50	* "	43.29
"	43.12	* "	37.71
"	36.00	"	42.00
"	42.50	"	41.71
"	35.00	"	40.71
"	33.75	"	38.29
"	36.25	"	38.28
Mixed callus	35.66	* "	36.00
"	39.83	"	33.00
"	39.67	"	41.75
"	38.50	"	34.00
"	32.00	"	33.12
"	47.00	"	33.75
"	45.12	"	42.37
"	43.26	"	36.12
"	43.13	"	39.17
"	34.00	"	35.62
"	43.62	"	41.37
"	41.12	"	34.47
* "	34.25	"	31.62
"	43.20	"	31.33
"	31.33	"	32.46
"	43.50	* "	33.87
"	32.50	"	34.87
"	35.25	"	32.37
"	31.88	"	48.37
* "	34.50	"	38.37

Appendix II.4. (continued)

ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)
Mixed callus	34.87
"	34.25
"	32.30
"	46.28
"	34.00
HG 2n→4n (1)	30.62
" (2)	29.37
" (3)	29.42
" (4)	30.17
" (5)	29.67
" (6)	29.62

Regenerants numbered (1) to (6) were selected as potential chimeras for further analysis and growth in vivo, as their stomatal lengths indicated a diploid nature whereas the chromosome numbers in the root-tips were that of tetraploid plants.

All those marked * are shown in Table 8.I, Section 8.

Appendix II.4. (continued)

B. Stomatal guard cell length measurements obtained from the leaves of plantlets regenerated from callus systems and autografts of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory

ORIGIN OF REGENERANT	MEAN STOMATAL LENGTH (μ)
Callus derived from stem tissue of S. sparsipilum	30.26
Callus derived from stem tissue of cv. Pentland Ivory	39.56
Autograft of S. sparsipilum	29.89
Autograft of cv. Pentland Ivory	39.24

These figures are shown in Table 8.1. $ection 8

Appendix II.4. (continued)

C. Stomatal guard cell length measurements obtained from the leaves of stock plantlets of S. sparsipilum cultured in vitro (Section 2.1)

STOMATAL GUARD CELL LENGTH (μ)	MEAN STOMATAL LENGTH
26, 28, 28, 28, 28, 28, 28, 30, 34, 34	29.2
26, 28, 28, 28, 30, 30, 30, 32, 32, 32	29.6
26, 28, 28, 28, 30, 30, 30, 30, 30, 32	29.2
28, 28, 30, 30, 30, 30, 32, 32, 32, 34	30.6
26, 26, 28, 28, 28, 28, 30, 30, 30, 32	28.6
24, 26, 26, 28, 28, 28, 30, 30, 32, 34	28.6
28, 28, 28, 28, 30, 30, 30, 32, 32, 32	29.8
30, 30, 30, 30, 30, 30, 30, 32, 32, 34	30.8
28, 30, 30, 30, 30, 30, 32, 32, 32, 34	30.8
28, 28, 28, 28, 28, 30, 30, 30, 30, 30	29.0
24, 24, 26, 26, 28, 30, 30, 30, 30, 30	27.8
26, 26, 26, 28, 28, 28, 28, 30, 32, 34	28.6
28, 28, 30, 30, 30, 30, 32, 32, 32, 34	30.6
26, 28, 30, 30, 30, 32, 32, 32, 32, 34	30.6
28, 28, 28, 30, 30, 30, 30, 32, 34, 34	30.4
26, 28, 28, 28, 30, 30, 30, 30, 32, 34	29.6
30, 30, 30, 32, 32, 32, 32, 32, 32, 34	31.6
26, 26, 26, 28, 28, 28, 30, 30, 32, 34	28.8
32, 32, 32, 32, 32, 34, 34, 34, 34, 36	33.2
28, 28, 32, 32, 32, 34, 34, 34, 36, 36	32.6

$\bar{\chi}$ = 30.00 ± 1.36μ

Variance = 1.838 Standard deviation = 1.356

99% Confidence Interval = $30.00 \pm 2.86 \times \frac{1.356}{\sqrt{20}}$

$= 30.00 \pm 0.87$

Appendix II.4. (continued)

C. Stomatal guard cell length measurements obtained from the leaves of stock plantlets of S. tuberosum ssp. tuberosum cv. Pentland Ivory cultured in vitro (Section 2.I.)

STOMATAL GUARD CELL LENGTH (μ)	MEAN STOMATAL LENGTH (μ)
36, 38, 38, 38, 40, 40, 40, 42, 42, 46	40.00
38, 38, 38, 38, 40, 40, 40, 42, 42, 42	39.8
34, 36, 36, 38, 38, 40, 40, 40, 40, 44	38.6
36, 36, 38, 38, 38, 38, 40, 40, 42, 42	38.8
36, 36, 38, 38, 40, 40, 40, 40, 40, 44	39.2
38, 40, 40, 40, 40, 40, 42, 42, 44, 46	41.2
38, 40, 40, 40, 40, 40, 42, 44, 44, 42	41.0
36, 38, 38, 40, 40, 40, 42, 42, 42, 44	40.2
36, 36, 36, 36, 38, 38, 38, 38, 40, 42	37.8
36, 36, 38, 38, 38, 38, 38, 40, 40, 40	38.2
34, 34, 36, 38, 38, 38, 38, 38, 40, 40	37.4
34, 36, 36, 36, 38, 38, 38, 40, 40, 40	37.6
36, 36, 36, 36, 38, 38, 40, 42, 42, 46	39.0
34, 36, 36, 36, 36, 38, 38, 38, 38, 40	37.0
34, 34, 36, 36, 36, 38, 38, 38, 40, 40	37.0
34, 36, 36, 36, 36, 36, 36, 38, 38, 40	36.6
36, 36, 38, 38, 38, 40, 40, 40, 40, 42	38.8
34, 36, 36, 38, 38, 38, 40, 40, 40, 42	38.2
36, 36, 36, 38, 38, 38, 40, 40, 40, 40	38.2
34, 34, 34, 36, 36, 38, 38, 38, 38, 40	40.0

$\overline{\chi}$ = 38.73 ± 1.29μ

Variance = 1.669 Standard deviation = 1.292

99% Confidence internal = $38.73 \pm 2.86 \times \frac{1.292}{\sqrt{20}}$

= $\underline{38.73 \pm 0.83}$

Appendix II.4. (continued)

D. Comparison of the stomatal guard cell length of the diploid species with that of the tetraploid species using the test statistic

$$t = \frac{\bar{y} - \bar{x}}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where s = pooled estimate of standard deviation

$$t = \frac{38.73 - 30}{0.4296\sqrt{\frac{1}{20} + \frac{1}{20}}} = \frac{8.726}{0.1358}$$

$$= 64.25$$

Using 't' tables with 38 degrees of freedom (n-1) + (n-1) this result (t = 64.25) is highly significant (α = <0.005)

Appendix II.5

A. Chromosome numbers obtained from the root-tips of plantlets regenerated from heterografts (HG) and mixed callus systems consisting of tissue derived from *S. sparsipilum* and *S. tuberosum* ssp. *tuberosum* cv. Pentland Ivory (Section 8.3)

ORIGIN OF REGENERANT	CHROMOSOME NUMBER	ORIGIN OF REGENERANT	CHROMOSOME NUMBER
HG 4n→2n	24	HG 2n→4n	48
"	24	"	48
"	24	"	48
"	24	HG 4n→2n	48
"	24	"	48
"	24	"	48
"	24	"	48
"	24	"	48
HG 2n→4n	48	"	48
"	48	HG 2n→4n	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48

Appendix II.5 (continued)

ORIGIN OF REGENERANT	CHROMOSOME NUMBER	ORIGIN OF REGENERANT	CHROMOSOME NUMBER
HG 2n→4n	48	HG 2n→4n	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
* "	46	"	48
"	48	"	48
"	48	"	48
"	48	"	48
HG 4n→2n	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
HG 2n→4n	48	"	48
"	48	"	48
* "	47	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
* "	47	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	* "	47
"	48	"	48
"	48	"	48

Appendix II.5. (continued)

ORIGIN OF REGENERANT	CHROMOSOME NUMBER	ORIGIN OF REGENERANT	CHROMOSOME NUMBER
HG 2n→4n	48	HG 2n→4n	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	* Mixed callus	46
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	* "	47
"	48	"	48
"	48	"	48

Appendix II.5. (continued)

ORIGIN OF REGENERANT	CHROMOSOME NUMBER	ORIGIN OF REGENERANT	CHROMOSOME NUMBER
HG 2n→4n	48	Mixed callus	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	* "	47
"	48	* "	47
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
Mixed callus	48	* "	46
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
"	48	"	48
* "	47	"	48
"	48	"	48
"	48	"	48
"	48	* "	47
"	48	"	48
"	48	"	48
"	48	"	48

Appendix II.5. (continued)

ORIGIN OF REGENERANT	CHROMOSOME NUMBER
*Mixed callus	47
"	48
"	48
"	48
"	48
"	48
"	48
HG 2n→4n (1)	48
" (2)	48
" (3)	48
" (4)	48
" (5)	48
" (6)	48

Regenerants numbered (1) to (6) were selected as potential chimeras for further analysis and growth in vivo, as their stomatal lengths indicated a diploid nature whereas the chromosome numbers in the root-tips were that of tetraploid plants.

All those marked * are shown in Table 8.1, Section 8.

Appendix II.5. (continued)

B. Chromosome numbers obtained from the root-tips of plantlets regenerated from callus systems, and autografts of S. sparsipilum and S. tuberosum ssp. tuberosum cv. Pentland Ivory, and stock plants cultured in vitro of S. sparsipilum and cv. Pentland Ivory

ORIGIN OF REGENERANT	CHROMOSOME NUMBER
Callus derived from stem tissue of S. sparsipilum	24
Callus derived from stem tissue of cv. Pentland Ivory	48
Autograft of S. sparsipilum	24
Autograft of cv. Pentland Ivory	48
Stock plant of S. sparsipilum	24
Stock plant of cv. Pentland Ivory	48

These figures are shown in Table 8.1. Section 8.

Appendix II.6.

A. Stomatal guard cell length measurements obtained from leaves of plants of S. sparsipilum cultured in vivo

STOMATAL GUARD CELL LENGTH (μ)	MEAN STOMATAL LENGTH (μ)
26, 30, 28, 28, 28, 28, 30, 30, 34, 32	29.4
26, 28, 28 28, 30, 30, 30, 32, 32, 32	29.6
28, 28, 28, 30, 30, 30, 30, 32, 34, 34	30.4
26, 28, 28, 28, 30, 30, 30, 32, 34, 34	30.00
28, 28, 28, 28, 30, 30, 30, 32, 32, 32	29.8
28, 28, 30, 30, 30, 30, 30, 32, 32, 34	30.4
26, 28, 30, 30, 30, 30, 32, 32, 32, 34	30.4
26, 28, 28, 28, 30, 30, 30, 30, 32, 34	29.6
28, 28, 28, 30, 30, 30, 32, 32, 32, 34	30.4
28, 28, 28, 28, 28, 30, 30, 32, 32, 32	29.6

$\bar{x}$ = 29.96 ± 0.38μ

Appendix II.6. (continued)

B. Stomatal guard cell length measurements obtained from leaves of plants of S. tuberosum ssp. tuberosum cv. Pentland Ivory cultured in vivo

STOMATAL GUARD CELL LENGTH (μ)	MEAN STOMATAL LENGTH (μ)
34, 36, 36, 38, 38, 40, 40, 40, 42, 42	38.6
36, 36, 36, 38, 38, 38, 40, 42, 42, 42	38.8
36, 38, 38, 38, 38, 40, 40, 40, 40, 44	39.2
36, 36, 36, 38, 38, 38, 40, 40, 40, 40	38.2
36, 38, 38, 38, 38, 38, 40, 40, 42, 42	39.0
36, 36, 38, 38, 38, 40, 40, 40, 40, 42	38.8
36, 36, 36, 36, 38, 38, 38, 40 40, 42	38.0
36, 36, 38, 38, 38, 38, 40, 40, 40, 42	38.6
36, 36, 36, 36, 36, 38, 38, 40, 42, 42	38.0
36, 36, 36, 38, 38, 40, 40, 40, 42, 42	38.8

$\overline{\chi}$ = 38.6 ± 0.39μ

Appendix II.6. (continued)

C. Stomatal guard cell length measurements obtained from leaves of plants derived from heterografts cultured in vivo

ORIGIN OF PLANT	STOMATAL GUARD CELL LENGTH (μ)	MEAN STOMATAL LENGTH
HG 2n→4n (1)	26, 26, 28, 28, 28, 30, 30, 30, 30, 30, 30, 30, 30, 30, 32, 32, 34, 34, 36, 32, 34, 36	30.3
HG 2n→4n (2)	26, 26, 28, 28, 28, 28, 28, 28, 28, 28, 28, 28, 30, 30, 30, 30, 32, 32, 34, 34	29.2
HG 2n→4n (3)	26, 28, 28, 28, 28, 28, 28, 28, 28, 28, 30, 30, 30, 30, 30, 30, 32, 32, 34, 34	29.5
HG 2n→4n (4)	26, 26, 26, 28, 28, 28, 28, 28, 28, 30, 30, 30, 30, 30, 32, 32, 32, 34	29.8
HG 2n→4n (5)	26, 26, 28, 28, 28, 28, 28, 28, 28, 30, 30, 30, 30, 30, 32, 32, 32, 34 34, 34	29.8

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